gp74 a Membrane Glycoprotein of the cis-Golgi Network that Cycles through the Endoplasmic Reticulum and Intermediate Compartment

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Abstract. A monoclonal antibody CC92 (IgM), raised against a fraction of rat liver enriched in Golgi membranes, recognizes a novel Endo H–resistant 74-kD membrane glycoprotein (gp74). The bulk of gp74 is confined to the cis-Golgi network (CGN). Outside the Golgi gp74 is found in tubulovesicular structures and ER foci. In cells incubated at 37°C the majority of gp74 is segregated from the intermediate compartment (IC) marker p58. However, in cells treated with organelle perturbants such as low temperature, BFA, and [AlF₄]⁻ the patterns of the two proteins become indistinguishable. Both proteins are retained in the Golgi complex at 20°C and in the IC at 15°C. Incubation of cells with BFA results in relocation of gp74 to p58 positive IC elements. [AlF₄]⁻ induces the redistribution of gp74 from the Golgi to p58-positive vesicles and does not retard the translocation of gp74 to IC elements in cells treated with BFA. Disruption of microtubules by nocodazol results in the rapid disappearance of the Golgi elements stained by gp74 and redistribution of the protein into vesicle-like structures. The responses of gp74 to cell perturbants are in sharp contrast with those of cis/middle and trans-Golgi resident proteins whose location is not affected by low temperatures or [AlF₄]⁻, are translocated to the ER upon addition of BFA, and stay in slow disintegrating Golgi elements in cells treated with nocodazol. The results suggest that gp74 is an itinerant protein that resides most of the time in the CGN and cycles through the ER/IC following the pathway used by p58.

STRUCTURE and function are intimately interrelated in the intricate machinery that is the Golgi complex (Farquhar and Palade, 1981; Dunphy and Rothman, 1985; Kornfeld and Kornfeld, 1985; Griffiths and Simons, 1986; Mellman and Simons, 1992). Recent results have provided new insights on how the Golgi complex is organized and functions. At least three compartments appear to constitute the Golgi complex: the cis-Golgi network (CGN)[1], the stack of cisternae, and the TGN (see the reviews by Rothman and Orci, 1992; and Mellman and Simons, 1992). The CGN appears to consist of the cis-most cisterna associated with an array of tubules (Lindsey and Ellisman, 1985; Rambourg

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1. *Abbreviations used in this paper:* BFA, brefeldin A; CGN, cis-Golgi network; Endo H, endo-β-N acetylglucosaminidase; GMP$_{c-1}$, cis-Golgi membrane protein; GMP$_{t-1}$, trans-Golgi membrane protein; IC, intermediate compartment; N-glycanase, peptide-N-glycosidase F; NRK, normal rat kidney; pAb, polyclonal antibody; PDI, protein disulfide isomerase; RAM, rabbit anti-mouse.

and Clermont, 1990) and is the Golgi subcompartment involved in receiving and sorting newly synthesized proteins from the ER (Palade, 1975; Pfeffer and Rothman, 1987). In most cells it also appears to be involved in the recycling of proteins and lipids to the ER (Pelham, 1988, 1989; Dean and Pelham, 1990; Pelham, 1991), the addition of the first N-acetylgalactosamine residues to O-linked oligosaccharides (Tooze et al., 1988), the phosphorylation of lysosomal enzymes (Pelham, 1988), and the fatty acylation of membrane proteins (Rizzolo and Kornfeld, 1988; Bonatti et al., 1989). The material transported from the ER arrives at the CGN via the intermediate compartment (IC) (Schweizer et al., 1988, 1990). The IC appears to be formed by a complex network of tubules that covers large areas of the cytoplasm. However, the nature of the IC remains controversial and it is not clear whether it is a distinct organelle interposed between the ER and the Golgi complex (Schweizer, A., G. Griffiths, T. Bachi, and H. P. Hauri. 1991. *J. Cell Biol.* 374:65a; Plutner et al., 1992) or if it is formed by the collection of ER outposts (i.e., transitional elements) involved in the exchange of materials with the Golgi complex (Hauri and Schweizer, 1992). The CGN and the ER/IC are connected

by vesicular organelles that shuttle and transport materials between the two (Palade, 1975).

Much remains to be understood on how the CGN and the IC are organized, function, and interact. Since organelles, as well as their subcompartments are defined by specific protein frameworks, our understanding of the structure and function of the CGN and the IC requires the characterization of their protein components to enable the study of their distribution and organization. This is presently limited by the paucity of accepted marker proteins (Hauri and Schweizer, 1992). Some studies have recently been reported with a few markers: the rat IC component p58 (Saraste et al., 1987; Saraste and Svensson, 1991) and its human homolog (Lahtinen et al., 1992) p53 (Schweizer et al., 1988, 1990); the KDEL receptor (Lewis and Pelham, 1992; Tang et al., 1993) and the small GTP-binding proteins rab2 (Chavrier et al., 1990); and rablbp (Plutner et al., 1991).

Here we report the characterization of a novel membrane protein, gp74, the bulk of which resides predominantly in the CGN and cycles between the Golgi and the ER/IC along the same pathway used by the IC marker p58. The responses of gp74 distribution to organelle perturbants such as BFA, low temperature, $[AlF_4]^-$ and nocodazol reveals the dynamics, morphology, and functional connections of a pathway between the CGN, the IC, and ER.

Materials and Methods

Cell Cultures

NRK (Normal rat kidney) cells were grown on plastic Petri dishes or glass coverslips, in 90% DME, 10% FCS, 10 mM morpholinoethane-sulfonic acid, 2 mM glutamine and antibiotics (50 U/ml penicillin; 50 μg/ml streptomycin) at 37°C (or room temperature), in an atmosphere of 93% air, 7% CO_2, and 85% humidity. When required the cells were incubated with BFA, a gift from Sandoz Laboratories (Basel, Switzerland), nocodazol (Sigma, St. Louis, MO), $[AlF_4]^-$, or the protein synthesis inhibitor cycloheximide.

Antibodies and Other Protein Probes

mAb CC29 was an IgM raised in a mouse injected with 0.1 M Na_2CO_3 treated membranes from a rat liver fraction enriched in the Golgi complex (Yuan et al., 1987). The development of mouse mAbs and rabbit polyclonal antibodies (pAb) to integral membrane proteins from the cis/middle (GMP$_{c-1}$) and trans-Golgi (GMP$_{t-1}$) has been previously reported (Yuan et al., 1987; Alcalde et al., 1992). The pAb to the IC marker p58 (Saraste et al., 1987) and the rat mAb to tubulin (Kilmartin et al., 1982) were gifts of Drs. Saraste (University of Bergen, Bergen, Germany) and Kilmartin (Laboratory of Molecular Biology, MRC, Cambridge, UK), respectively. The pAb to the ER marker PDI (Lambert and Freedman, 1985) was a gift of Dr. Castaño (Instituto de Investigaciones Biomédicos, CSIC, Madrid, Spain). The mAb Cu-1 to the Tn epitope (GalNAcα-O-Ser/Thr) (Takahashi et al., 1988) was a gift of Dr. S. I. Hakomori (The Biomembrane Institute, Seattle, WA). The pAb to mannosidase II (Moremen and Touster, 1985) was a gift of Dr. M. Baron (Center for Biotechnology, Karolinska Institute, Huddinge, Sweden). Limax flavus agglutinin (LFA) was from Calbiochem-Novabiochem Corp. (La Jolla, CA). Protein A was from Pharmacia (Lund, Sweden). Rhodamine and fluorescein-conjugated rabbit anti-mouse IgG + IgM (RAM) pAb was from Dako (Dakopatts, Glostrup, Denmark). Rabbit anti-mouse IgG and IgM pAbs conjugated to gold were from BioCell (Cardiff, UK). Biotin-conjugated goat anti-rabbit IgG + IgM (RAM) pAb and fluorescein-conjugated streptavidin were from Boehringer Mannheim Biochemicals (Indianapolis, IN) and were used to stain p58 (Lahtinen et al., 1992).

Characterization of gp74

Incubation of NRK extracts, metabolically labeled with $[^{35}S]$methionine,

with antibody CC92, did not immunoprecipitate any protein and the antibody failed also to react with proteins from either rat liver or NRK extracts resolved by SDS-PAGE and blotted onto nitrocellulose. The identification of gp74 was thus achieved by photoaffinity labeling using sulfosuccinimidyl 2-(p-azido salicyl-amido) ethyl-1',3'-dithiopropionate (SASD) (Tae and Ji, 1982) attached to mAb CC92. Briefly, 5.5 μg of the photoaffinity reagent SASD (Pierce, Rockford, IL) was labeled with 0.5 mCi ^{125}I to a maximal specific activity of 5×10^4 Ci/mol and conjugated to 36 μg of CC92 antibody, according to the manufacturer instructions. NRK cells, grown to 90% confluence in a 100 mm dish were fixed-permeabilized for 5 s with 90% methanol, at room temperature, to permeabilize the cells without irreversible fixation of the proteins. The fixed-permeabilized cells were then incubated for 1 h at 37°C in the dark with the $[^{152}I]$ SASD-antibody conjugate in PBS, in the absence or presence of a 200-fold molar excess of unconjugated CC92 antibody. After thorough washing of the cells with PBS, the SASD was photoactivated for 15 min at a wavelength between 244–366 nm. The $[^{125}I]$ SASD antibody conjugate covalently bound to the protein was reduced for 30 min at 37°C with 50 mM DTT in PBS, washed quickly twice with PBS, and incubated with 250 μl of 10 μg/ml RNAse, 10 μg/ml DNAse in PBS for 30 min at 37°C. Finally, the cells were harvested, and boiled for 20 min in the RNAse/DNAse solution adjusted to contain 2% SDS, 0.1 M DTT, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml chymostatin, 1 μg/ml aprotinin. ^{125}I-labeled proteins were analyzed by SDS-PAGE. Identical experiments were also performed with $[^{125}I]$ SASD-conjugated $F(ab')_2$ CC92 fragments. These $F(ab')_2$ fragments were produced by incubating purified CC92 with pepsin (Boehringer Mannheim Biochemicals), at a wt/wt ratio of 25/1, in 35 mM sodium acetate/150 mM NaCl, for 24 h at 4°C; the fragments were separated from undigested CC92 antibody by gel filtration on a column of Superose (Pharmacia, FPLC) and 0.26 nmol coupled to 16 nmol $[^{125}I]$ SASD as described above.

Protein electrophoresis, autoradiography, and glycoprotein analysis were performed as previously described (Barriocanal et al., 1986). Studies on the partition of gp74 between the Triton X-114 and aqueous phases were performed as described (Bordier, 1981).

Immunofluorescence Microscopy

Single and double immunofluorescence microscopy studies were performed on NRK cells as described (Barriocanal et al., 1986; Yuan et al., 1987; Lahtinen et al., 1992).

Immunoelectron Microscopy

Monodisperse colloidal gold particles with average diameters of 15, 8, or 10 nm were prepared as described (Frens, 1973; Slot and Geuze, 1985) and conjugated to protein A (Roth, 1982) and fetuin (Roth et al., 1984) as reported. Male Sprague-Dawley rats were anesthetized, perfused with balanced salt solution and then for 20 min with 0.1 M phosphate buffer (pH 7.4) containing either 4% paraformaldehyde, or 4% paraformaldehyde/0.1–0.05% glutaraldehyde. Duodenum, distal, and proximal colon were quickly excised, cut into small pieces, and immersed in the respective fixatives for 2 h at room temperature. NRK cells were cooled on ice, washed with either ice-cold 0.1 M sodium cacodylate, pH 7.2, or 0.2 M Hepes, pH 7.2, detached from the culture dishes by digestion with 25 μg/ml proteinase K, resuspended with vigorous pipetting, pelleted by centrifugation at 3,000 rpm for 1 min, and immersed in the fixatives described above. Fixed tissues and cells were rinsed in the same buffer and free aldehyde groups blocked with 50 mM ammonium chloride in PBS for 1 h at room temperature, with several buffer changes. The fixed specimens were embedded in Lowicryl K4M at −35°C (Carlemaln et al., 1982), and postembedding immunogold labeling done on either Lowicryl K4M ultrathin sections (Roth, 1982) or ultrathin cryosections (Slot and Geuze, 1985).

Single Labeling with mAbs or pAb. Thin sections were floated on drops of PBS for 10 min, at room temperature, and then incubated for 2 h at room temperature, or overnight at 4°C, on small droplets of mAbs in PBS, or sera in PBS/1% skim milk. After rinsing twice with PBS, sections were incubated with mAbs for 45 min at room temperature with 25 μg/ml affinity-purified RAM IgG + IgM in PBS/0.1% Tween 20. Sections incubated with RAM or with 15C8 antiserum were floated for 1 h at room temperature on a droplet of protein A-gold (15 nm) (OD_{525} = 0.4) in PBS^{3+} (PBS/1% BSA/0.075% Triton X-100/0.075% Tween 20), washed twice with PBS, once with distilled water, and air dried.

Double Labeling with Limax flavus Lectin and mAbs to Tn and gp74. The first labeling reaction was performed with the lectin, according to the two step cytochemical procedure described by Roth et al. (1984): briefly, sections were floated on a droplet of PBS and then transferred to a droplet

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of LFA (75–100 μg/ml in PBS), washed and incubated with fetuin–gold (8 nm) (OD_{525} = 0.3) in PBS^{3+} for 30 min. The labeled sections were rinsed twice in PBS, floated on 1% glutaraldehyde in PBS for 15 min, washed again in PBS, and laid onto a drop of 50 mM of ammonium chloride in PBS for 60 min to block free aldehyde groups.

Following two more washes in PBS, the sections were incubated with the corresponding mAbs, either anti-Tn or anti-gp74, which were labeled with RAM and then with protein A–gold (15 nm).

Double Labeling with Antibodies. For double labeling with mAbs Cu-1 and CC92, the tissue sections were incubated first with mAb Cu-1 and then with RAM IgG–gold (10 nm) diluted to 1/10 or 1/20 in PBS. The sections were fixed with 1% glutaraldehyde, and free aldehyde groups quenched with ammonium chloride (50 mM in PBS) before incubation with mAb CC92 and, subsequently, with RAM IgM (15 nm) diluted 1/10–1/20 in PBS. For double labeling with either anti-GMP$_{c-1}$ or anti-PDI serum and mAb CC92, the sections were incubated first with either serum, labeled with protein A–gold (10 nm), fixed with glutaraldehyde, and quenched with ammonium chloride before incubation with mAb CC92. Labeling of mAb CC92 was visualized directly using RAM IgM–gold (15 nm) or RAM followed by protein A–gold. For double labeling with anti-p58 serum and mAb CC92 the sections were incubated first with the antibody to p58, labeled with goat anti-rabbit IgG–gold (15 nm), fixed with glutaraldehyde, and quenched with ammonium chloride, before incubation with mAb CC92 and subsequently with RAM IgM–gold (10 nm).

All the gold-labeled sections were air-dried and stained in 3% uranyl acetate for 6 min and with 1% lead citrate for 45 s in a nitrogen atmosphere, before viewing in a Phillips 301 electron microscope at 60 KV electron acceleration voltage.

Other Procedures

The metabolic labeling of NRK cells, preparation of cell extracts, immunoprecipitations, gel electrophoresis, and immunoblotting were performed as previously described (Bonifacino et al., 1985; Barriocanal et al., 1986; Alcalde et al., 1992). The time courses of the redistribution of gp74, p58, GMP$_{c-1}$, GMP$_{t-1}$ in response to BFA, $[AlF_4]^-$, low temperatures and nocodazol, were studied by quantifying the effects of the drugs on groups of 100 cells stained and studied by immunofluorescence microscopy.

Other Reagents

Tetrachloroauric acid, trisodium citrate, polyethylene glycol (20,000 mol wt), paraformaldehyde, Tween 20, ammonium chloride, aluminium chloride, and sodium fluoride were from Merck (Darmstadt, FRG); glutaraldehyde (25% in water) was from Fluka (Buchs, Switzerland); Triton X-114 and fetuin were from Sigma.

Results

Antibody CC92 Recognizes a 74-kD Membrane Glycoprotein

Since antibody CC92 failed to immunoprecipitate or react with the antigen on immunoblots, the characterization of the antigen was achieved by photoaffinity labeling using [^{125}I] SASD conjugated to the CC92 antibody. When [^{125}I] SASD antibody was incubated with cells fixed–permeabilized with methanol at room temperature for 5 s, a 74-kD protein (gp74) was radiolabeled (Fig. 1, lane *1*). The specificity of the reaction was shown by the lack of labeling when the incubation was performed in the presence of a 200-fold molar excess of unconjugated antibody (Fig. 1, lane *2*) but not by excess of an unrelated IgM (not shown). The same protein was labeled by [^{125}I] SASD-CC92 (Fab')$_2$ fragments (Fig. 1, lane *5*) and the reaction again was inhibited by performing the incubation in the presence of a 200-fold molar excess of unconjugated CC92 antibody (lane *6*). The weaker reaction of gp74 with [^{125}I] SASD-CC92 (Fab')$_2$ fragments than with intact [^{125}I] SASD-CC92 IgM, is probably due to the more extensive cross-linking and consequent inactivation of the

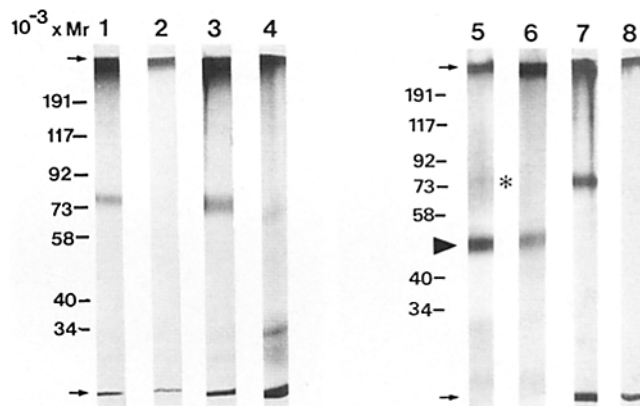*Figure 1.* Antibody CC92 recognizes a 74-kD membrane glycoprotein. NRK cells fixed-permeabilized for 5 s with methanol were incubated with the [^{125}I]-labeled SASD-CC92 antibody conjugate and the affinity-labeled proteins analyzed by SDS-PAGE and autoradiography (lane *1*). Labeling as in lane *1* but incubation performed in the presence of a 200-fold molar excess of unconjugated antibody CC92 (lane *2*). Digestion of labeled gp74 with Endo H (lane *3*) and with N-glycanase (lane *4*). gp74 (*) radiolabeled by [^{125}I]-labeled SASD-CC92 F(ab')$_2$-conjugated fragments (lane *5*). Labeling as in lane *5* but in the presence of a 200-mol excess of cold CC92 antibody (lane *6*). The protein marked with the arrowhead corresponds to SASD cross-linked F(ab')$_2$ CC92 fragments, separated by the reducing conditions used in the electrophoresis. Partition of gp74 between the Triton X-114 (lane *7*) and the aqueous (lane *8*) phases. The top and bottom of the gels are marked with arrows.

fragments by SASD as suggested by the weaker fluorescent staining of cells incubated with CC92 (Fab')$_2$ treated with SASD (not shown). This might explain the presence of a strong 47-kD band (Fig. 1, lanes *5* and *6*) which showed the molecular weight expected for IgM (Fab')$_2$ fragments produced by pepsin digestion (Pascual and Clem, 1992) and comigrated with [^{125}I] SASD-CC92 (Fab')$_2$ fragments (not shown).

Analysis of the sensitivity of gp74 to endo H and N-glycanase digestion revealed that the protein contained fewer N-linked high-mannose residues than complex N-linked carbohydrates (Fig. 1, lanes *3* and *4*). The polypeptide backbone had an apparent molecular weight of 34 kD, indicating a surprisingly high number of complex carbohydrates in gp74 (see Discussion). Finally, incubation of gp74 with Triton X-114 at 37°C (Bordier, 1981) resulted in complete partitioning of the protein into the detergent phase (Fig. 1, compare lanes *7* and *8*), indicating that gp74 was a membrane glycoprotein.

gp74 Is Localized in the Golgi Complex and Punctate Structures Clustered in the Golgi Area

NRK cells fixed–permeabilized with cold (−20°C) methanol, when incubated with the antibody to gp74 displayed the fluorescence pattern shown in Fig. 2 *A*. There was an intense staining of a large reticulum, located at one of the poles of the nucleus, and numerous punctate structures distributed in its vicinity. The reticulum was identified as part of the Golgi complex by double staining of cells with antibodies to gp74 and to the Golgi integral membrane proteins GMP$_{c-1}$ (compare Fig. 2, *A* and *B*) and GMP$_{t-1}$ (not shown). As expected
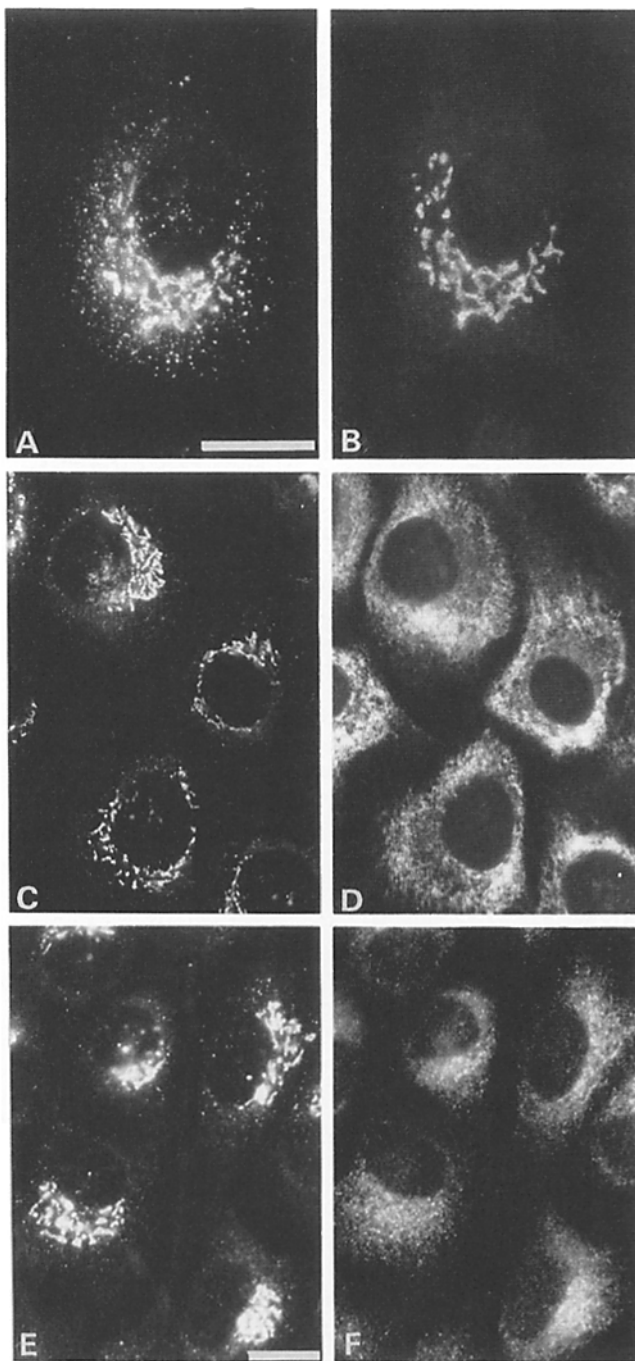
Figure 2. Cellular distribution of gp74. NRK cells double stained with antibodies to gp74 (*A*) and GMP$_{c-1}$ (*B*), to gp74 (*C*) and PDI (*D*) and to gp74 (*E*) and p58 (*F*). Note the staining of the Golgi reticulum and punctate structures by gp74, the localization of the Golgi to the area of partial ER (i.e., PDI) exclusion and the different distributions of gp74 and p58. Bars, 15 μm.

for the Golgi, the reticulum was located in the area of partial ER exclusion as seen in cells double stained for gp74 and the ER marker protein disulphide isomerase (see Fig. 2, *C* and *D*). Moreover, the distribution of gp74 was markedly different from that of the IC marker p58 which stained a granuloreticular structure which was spread beyond the Golgi area (Saraste and Svensson, 1991) and stained the

Golgi complex with less intensity than gp74 (Fig. 2, compare *E* and *F*).

EM Studies Localizes gp74 to the cis-most Golgi, Smooth Tubulo-vesicular Structures and Discrete Zones of the ER

The localization of gp74 within the Golgi complex was studied at the ultrastructural level on intestinal goblet cells, which display a highly polarized Golgi complex between a well developed ER, on the *cis* side, and clusters of mucin droplets, on the *trans* side. The polarization of the Golgi complex was demonstrated by the complete segregation of simple O-linked glycans (GalNacα-O-Ser/Thr) in the *cis*-Golgi from sialylated saccharides in the *trans*-Golgi, in cells double labeled with the mAb Cu-1 (15 nm gold) and Limax flavus lectin (8 nm gold), respectively (Fig. 3 *A*). The distribution of gp74 within the Golgi complex was localized to a compartment opposite to the trans-Golgi, as demonstrated by the segregation of the antibody to gp74 (15 nm gold) from the Limax flavus lectin (8 nm gold) (Fig. 3 *B*). Furthermore, gp74 (15 nm gold) showed partial colocalization both with the *cis*/middle-cisterna marker GMP$_{c-1}$ (10 nm gold) (Fig. 3 *C*) and the simple O-linked glycans in the *cis*-Golgi (not shown). A detailed comparison of the distributions of gp74 and GMP$_{c-1}$ revealed that gp74 was slightly more cis than GMP$_{c-1}$ (Table I *A*). The cisterna housing gp74 was frequently swollen and displayed the fenestrated morphology characteristic of the *cis*-most cisterna (Fig. 3 *B*) (Rambourg et al., 1979; Rambourg and Clermont, 1990). The data was consistent with the accumulation of gp74 in the *cis*-most Golgi (i.e., CGN).

In NRK cells gp74 also accumulated in the *cis* side of the Golgi (Fig. 4 *A*). The distribution of gp74 outside the Golgi complex was further studied by EM in these cells also labeled for the ER marker protein disulphide isomerase (PDI) and the IC marker p58. The studies, performed in the absence and presence of cycloheximide (not shown), showed that gp74 was localized in clusters at discrete and separate ER foci (Figs. 4, *B* and *G*), as well as in long tubules (Fig. 4, *D* and *E*) and 50–60 nm vesicles (Fig. 4 *F*). The areas of ER (Fig. 4 *B*) and some of the tubules and vesicles labeled with gp74 were frequently found to contain PDI (Fig. 4 *C*, *E*, and *F*). Moreover, gp74 overlapped partially with the IC marker p58 in the ER foci (Fig. 4, *G* and *H*) and in electrolucent pleomorphic structures with a light electrodense core (Fig. 4 *I*). A quantitative analysis of the gp74 and p58 distributions indicated a preferent localization of gp74 in the Golgi and of p58 in the ER (Table I *B*).

Changes in gp74 and p58 Distributions with Temperature Shifts

The concentration of gp74 in the CGN and its presence in ER and tubulovesicular structures suggested the possibility that gp74 was a protein that cycled between the Golgi complex and the ER. Furthermore, the partial overlapping between the distributions of gp74 and the IC marker p58 suggested that the two proteins may use the same pathway of cycling. To test this possibility, the distributions of gp74 and p58 were compared in cells incubated at 37, 20, and 15°C, temperatures that alter the transport through the exocytic pathway (Saraste et al., 1986; Kuismanen and Saraste, 1989;
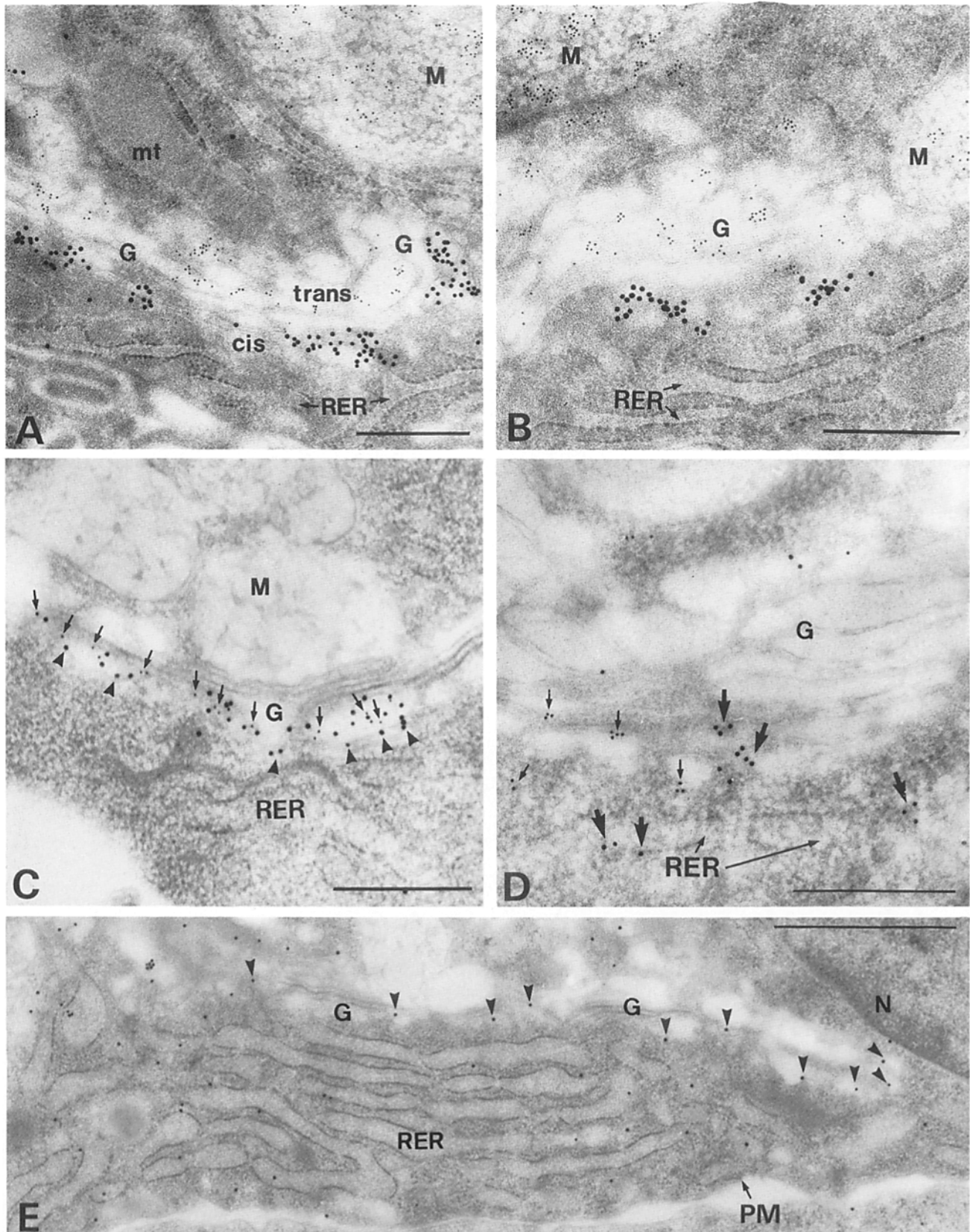
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Figure 3. Localization of gp74 within the Golgi complex. Lowicryl sections of rat goblet cells. (*A*) Labeling of the *cis* (antibody to Tn antigen, 15 nm gold), and *trans* (sialic acid residues labeled with Limax flavus lectin/fetuin, 8 nm gold) sides of the Golgi complex. (*B*) Segregation of gp74 (antibody CC92, 15 nm gold) and sialic acid residues (Limax flavus lectin/fetuin, 8 nm gold). (*C*) Distribution of gp74 (antibody CC92, 15 nm gold) and GMP$_{c-1}$ (antibody 15C8, 10 nm gold), respectively. Note the slightly *cis* localization of gp74 (*arrowheads*) with respect to GMP$_{c-1}$ (*arrows*). (*D*) Distribution of gp74 (antibody CC92, 10 nm gold, *small arrows*) and p58 (antibody to p58, 15 nm gold, *large arrows*): note the similar location of both antigens and their segregation into different clusters. (*E*) Distribution of p58 throughout the *cis*-Golgi (*arrowheads*) and ER cisternae. *G*, Golgi complex; *M*, mucin droplets; *mt*, mitochondria; *N*, nucleus; *PM*, plasma membrane. *RER*, rough endoplasmic reticulum. Bars, 0.5 μm.
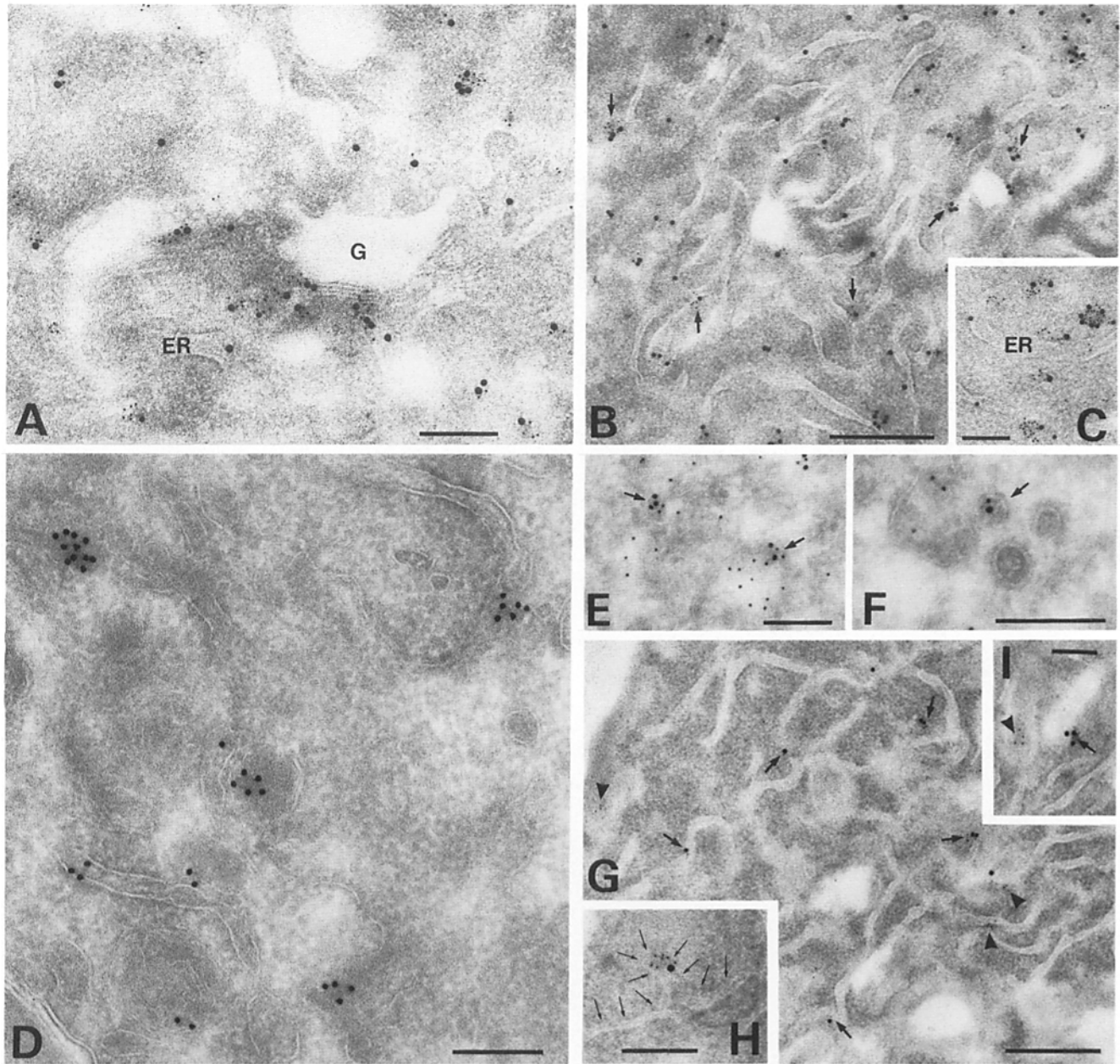
Figure 4. Localization of gp74 outside the Golgi complex. Lowicryl sections of NRK cells (*A-C, G-I*) and ultra-thin cryosections of rat enterocytes (*D-F*) labeled for gp74 (*A-C, G-I*, 10 nm gold; *D-F*, 15 nm gold), PDI (*A-C*, 15 nm gold; *E* and *F*, 10 nm gold) and p58 (*G-I*, 15 nm gold). (*A*) Note the clusters of gp74 and PDI in the *cis* side of the Golgi (*G*). (*B* and *C*) Observe the foci distribution of gp74 clusters through the ER cisternae labeled with PDI (*arrows*). (*D* and *E*) Clustering of gp74 within long tubules that contain PDI (*E, arrows*). (*F*) Vesicle labeling by gp74 and PDI (*arrow*). Labeling of ER cisternae (*G*), sacks (*H; arrows* mark the contour of tubules ending in a sack) and a pleomorphic electrolucent structure (*I*) by clusters of gp74 and p58 (*arrows*) and gp74 alone (*arrowheads*). Bars: (*A-F, H* and *I*) 200 nm; (*B* and *G*) 500 nm.

Schweizer et al., 1990). Comparison of cells incubated at 37°C with cells incubated at 20°C revealed no significant effect of the low temperature on the gp74 distribution (compare Figs. 2 *E* and 5 *A*). In contrast, the incubation at 20°C resulted in a dramatic accumulation of p58 in the Golgi complex (compare Fig. 5 *B* with Figs. 2 *F* and 5 *A*). However, in cells incubated at 15°C, gp74 was found to stain less the Golgi complex and to overlap more extensively with p58 than at 37°C (Fig. 5, compare *C* to *F*). The distribution of gp74 at 15°C was similar to that of p58 in cells incubated at

37°C, as shown also by EM studies that localized the two proteins in ER foci and electrolucent pleomorphic vesicles (data not shown). Finally, the change in gp74 distribution was also in contrast with the insensitivity of the resident *cis*-Golgi protein, GMP_{c-1}, to incubation at 15°C temperature (Fig. 5 *G*).

The results identified gp74 as a putative itinerant Golgi protein. They suggested that at 20°C transport from the Golgi to the IC was slowed down resulting in the retention of p58 in the Golgi, and at 15°C transport from the IC to

Table I. Comparison of the Distributions of gp74, the cis-Golgi Markers GMP_c-1 and Tn, and the IC Marker p58 through the Golgi and ER

Antigen						
A	-1	+1	+2	+3	+4	+5
gp74	26 ± 2	30 ± 3	18 ± 2	5 ± 1	–	–
p58	7 ± 1	11 ± 3	2 ± 1	–	–	–
GMP_c-1	4 ± 1	11 ± 1	13 ± 2	2 ± 1	–	–
TN	30 ± 3	39 ± 4	29 ± 2	8 ± 2	–	–

B	Endoplasmic Reticulum	Golgi complex
gp74	4 ± 1	64 ± 8
p58	10 ± 1	19 ± 2
GMP_c-1	0.2 ± 0.1	29 ± 2
Tn	0.3 ± 0.1	98 ± 3

The distribution of the antigens was studied by counting the gold particles on micrographs from Lowicryl sections of rat goblet cells immunostained as indicated in Fig. 3. (*A*) Distribution of antigens within the Golgi. Individual Golgi cisternae were numbered in a *cis* to *trans* direction from +1 to +5. Position −1 corresponds to gold particles found in pre-Golgi elements adjacent to the cis-most cisterna (+1). (*B*) Distribution of antigens between the Golgi and the ER in rat goblet cells.

the Golgi was blocked (Schweizer et al., 1990; Saraste and Svensson, 1991) causing the retention of gp74 and p58 in the IC. The codistribution of gp74 and p58 at 20° and 15°C also suggests that both proteins use the same Golgi-ER/IC cycling pathway.

BFA Induces the Translocation of gp74 to p58 Positive Vesicles

Studies in cells treated with BFA have shown marked differences in the redistribution of *cis*-Golgi and IC proteins, with the *cis*-Golgi proteins translocated to the ER (Lippincott-Schwartz et al., 1989; 1990; Doms et al., 1989; Ulmer and Palade, 1989; Reeves and Banting, 1992; Alcalde et al., 1992) and the bulk of p58 retained in what appears to be IC-derived or transport elements (Lippincott-Schwartz et al., 1990; Saraste and Svensson, 1991). The apparent cycling of the Golgi gp74 through the IC made it interesting to study if its response to BFA was influenced by its access to IC elements. In cells treated for 10–15 min with BFA the *cis*-Golgi marker GMP_c-1 showed a uniform distribution consistent with its translocation to the ER (Alcalde et al., 1992) (Fig. 6 *A*). The bulk of gp74 was however found in p58 positive punctate structures scattered throughout the cytoplasm (Fig. 6, compare *B* and *C*). Studies of the BFA-treated cells by EM confirmed the disappearance of the Golgi complex (not shown) and the presence of gp74 in pleomorphic vesicles (average large axis 540 nm) that devoid of the ER marker PDI (Fig. 7, *A* and *B*) frequently contained p58 (Fig. 7, *C* and *D*). In addition, as in untreated cells, gp74 was also localized in discrete foci within the membrane of swollen ER cisternae (Fig. 7, *A* and *C*) (see Discussion).

The Golgi Elements Stained by gp74 and GMP_c-1 Are Reassembled at Different Rates upon Removal of BFA

A previous study on the reassembly of the Golgi complex in cells pretreated with BFA has shown that the removal of the drug was followed by the rapid translocation of GMP_c-1 from the ER into vesicle-like structures, but that reassembly of the *cis*-Golgi was a much slower process that required nearly 1 h (Alcalde et al., 1992). The different redistribution of gp74 and GMP_c-1 in response to BFA and the EM observation that gp74 and GMP_c-1 are probably in distinct *cis*-Golgi compartments made it interesting to compare the reassembly of the Golgi by following the distributions of gp74 and GMP_c-1. The reconstruction of the Golgi elements stained by gp74 (Fig. 6 *D*) occurred between 8 and 10 min after removal of the drug, while GMP_c-1, though transported out of the ER, was retained in vesicle-like structures for an hour before its incorporation into the Golgi (see Fig. 6, *F* and *G*). The rapid incorporation of gp74 into a Golgi reticulum and the lag between the release of GMP_c-1 from the ER and its incorporation into Golgi elements are all consistent with the location of gp74 and GMP_c-1 in distinct Golgi compartments which are reassembled at different rates (Alcalde et al., 1992) (see Discussion).

[AlF_4]− Induces the Redistribution of gp74 from the Golgi to p58 Positive Vesicles in Cells That Show No Change in the Distribution of Golgi Resident Proteins

Previous studies have shown that non-clathrin–coated vesicles are involved in the transport of materials between the ER to and through the Golgi complex (Malhotra et al., 1989; Orci et al., 1986, 1989; Serafini et al., 1991; Peter et al., 1993) and that stabilization of their coats by [AlF_4]− results in vesicle accumulation and inhibition of transport (Melancon et al., 1987; Beckers et al., 1987; Beckers and Balch, 1989; Donaldson et al., 1991; Peter et al., 1992).

Since gp74 appeared to cycle between the Golgi complex and the IC, we sought to confirm its itinerant character by studying its distribution in cells treated with [AlF_4]−. Control experiments included [AlF_4]−-treated cells stained for the immobile Golgi proteins GMP_c-1 and GMP_t-1 as well as for the itinerant IC p58. As shown in Fig. 8, cells that were treated with 50 μM [AlF_4]− for more than 20 min, when double stained for gp74 and either GMP_c-1 or GMP_t-1, displayed gp74 in punctate structure clustered in the Golgi area and GMP_c-1 and GMP_t-1 in intact Golgi reticulums (Fig. 8, compare *A* to *C*). In cells double stained for gp74 and p58 it was noted that incubations with [AlF_4]− for periods shorter than 20 min resulted in concentration of p58 in the Golgi complex (compare Figs. 2 *F* with 8, *E* and *G*). However, in incubations longer than 20 min [AlF_4]− caused the disappearance of the Golgi staining and the appearance of punctate structures which were stained by gp74 (Fig. 8, *H* and *I*). The punctate structures were identified by EM as 40–70 nm electrodense vesicles (Fig. 8 *J*). Again, the results were consistent with the itinerant character of gp74 (see Discussion).

[AlF_4]− Does Not Interfere with the Redistribution of gp74 in BFA-treated Cells but Delays the Translocation of Golgi Resident Proteins to the ER

It has been reported that [AlF_4]− blocks the BFA induced translocation of *cis*-Golgi proteins to the ER (Donaldson et al., 1991) and the redistribution of the procoat component β-COP (Ktistakis et al., 1992). We thus studied the effect of [AlF_4]− on the redistribution of gp74 in cells treated with
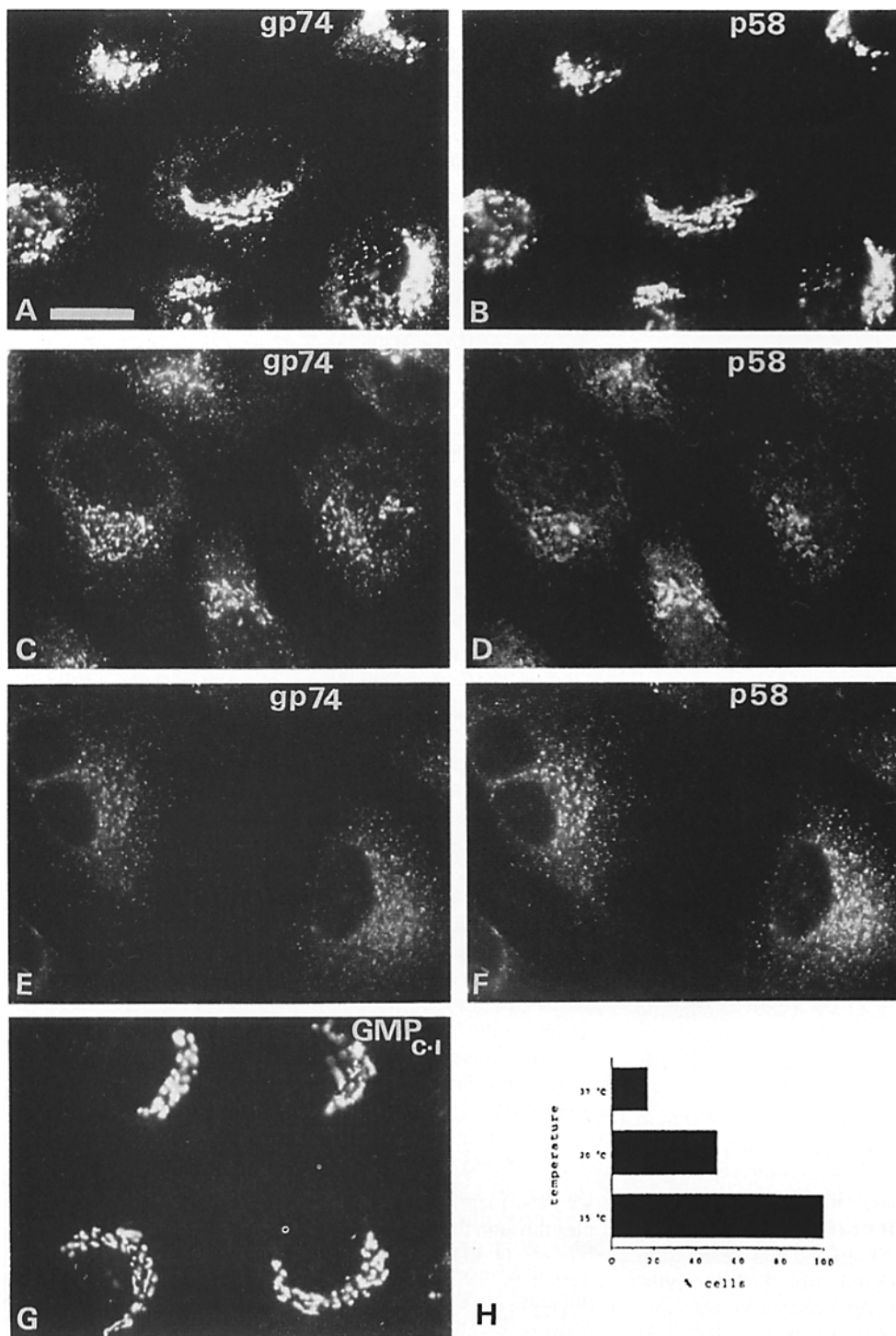
Figure 5. Redistribution of gp-74 and p58 in cells incubated at low temperatures. NRK cells were incubated at 20°C for 2 h (*A* and *B*) or at 15°C for either 30 min (*C* and *D*) or 2 h (*E-G*). Cells double stained for gp74 (*A, C,* and *E,* rhodamine channel) and p58 (*B, D* and *F,* fluorescein channel) and single stained for GMP_{c-1} (*G*). Note the retention of gp74 and p58 in the Golgi of cells incubated at 20°C (*A* and *B*) and their gradual redistribution to granular structures in cells incubated at 15°C (*C-F*). Observe that the distribution of GMP_{c-1} is not affected by the low temperature incubation (*G*). (*H*) Time courses of the redistributions of gp74 and p58 at 15°C and 20°C: percentage of cells showing colocalization of gp74 and p58 at different temperatures. Bars, 15 μm.

BFA. Preincubation of the cells in 50 μM $[AlF_4]^-$ for 10 min did not prevent the translocation of the protein to vesicle-like structures (Fig. 9, *A* and *B*) though markedly delayed the disassembly of Golgi elements stained by GMP_{c-1} (Fig. 9, *C* and *D*), α-mannosidase (Fig. 9, *E* and *F*) or GMP_{t-1} (for the time course of translocation of the four proteins see Fig. 9 (*G* and *H*).

The gp74 Undergoes a Rapid Redistribution in Response to Microtubule Depolymerization

Previous studies have shown that the Golgi complex under-

goes a relatively slow disorganization upon changes in microtubule organization, thus implicating microtubules in the maintenance of the Golgi structure and its centrosomal localization (Kupffer et al., 1982, 1983; Wehland et al., 1983*a,b*; Sandoval et al., 1984; Turner and Tartakoff, 1989; Kreis, 1990; Corthésy-Theulaz, 1992). Microtubules have also been implicated in the traffic of vesicles that shuttle between the ER, IC and Golgi complex (Lippincott-Schwartz et al., 1990; Saraste and Svensson, 1991). The effect of microtubule disruption on the distribution of gp74 was examined in cells that were treated with 20 μM nocodazol and, then, double stained for gp74 and the Golgi resident proteins GMP_{c-1}
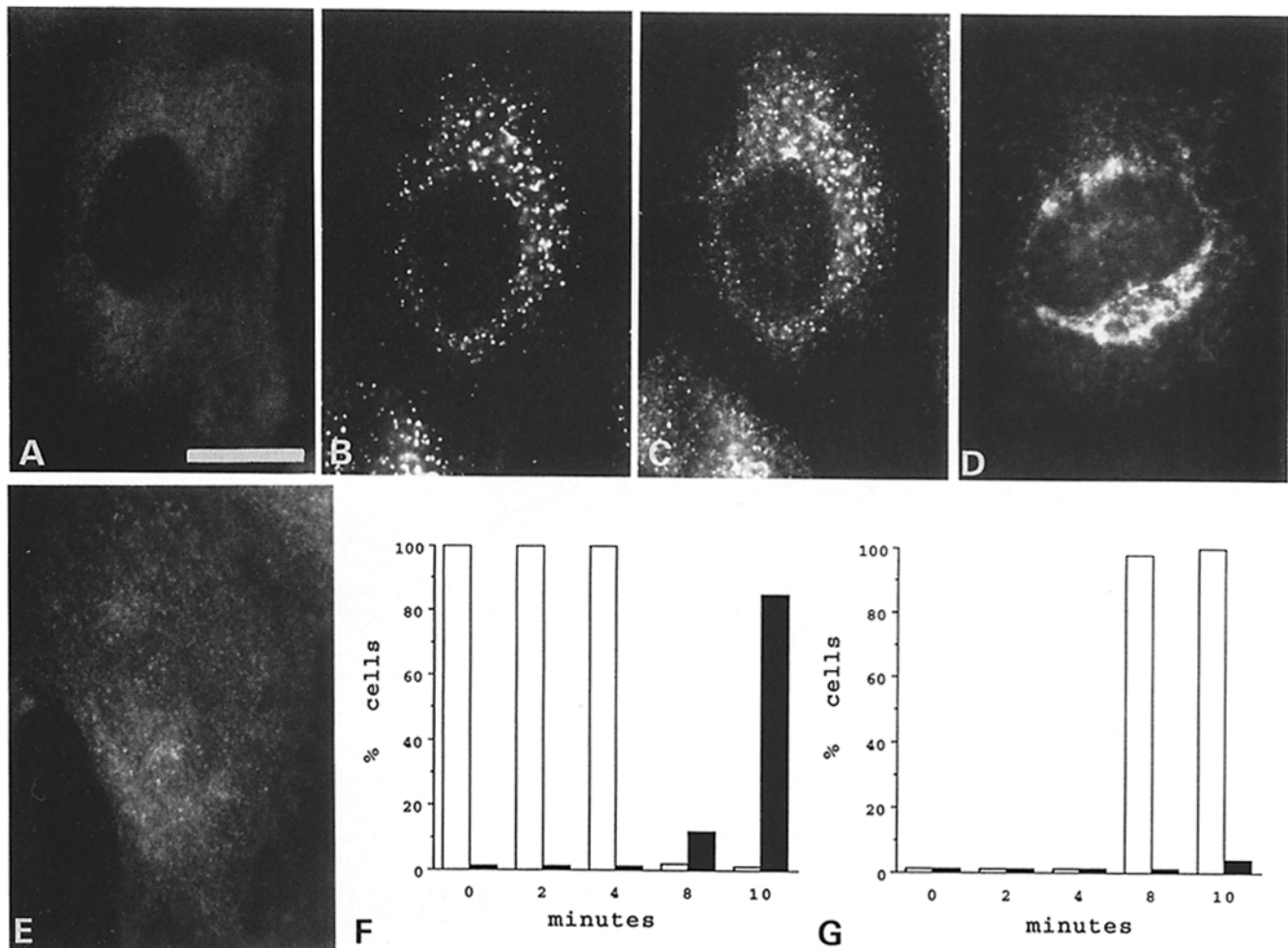
Figure 6. Changes in gp74 distribution upon addition and removal of BFA. NRK cells were incubated for 10 min with 1 µg/ml BFA. (*A*) Cell stained for GMP_{c-1}. (*B* and *C*) Cell double stained for gp74 (*B*) and p58 (*C*). Note the diffuse cytoplasmic staining of the cell stained for GMP_{c-1} (compare 6 *A* and with Fig. 2 *B*) and the colocalization of gp74 and p58 to punctate structures (*B* and *C*). Cells incubated for 70 min with 1 µg/ml BFA and then for 10 min in drug-free medium were stained for gp74 (*D*) or GMP_{c-1} (*E*). Time courses of the redistribution of gp74 (□) and GMP_{c-1} (■) upon removal of BFA: cells displaying the proteins in punctate structures (*F*) and in fully reconstructed Golgi complexes (*G*). Note that the fast rise in gp74-positive punctate structures is followed by a rapid decline that coincides with the assembly of Golgi elements. Observe that GMP_{c-1} is slowly released from the ER and is not incorporated into Golgi elements. Bars, 15 µm.

and GMP_{t-1}. Depolymerization of microtubules (Fig. 10, *A* to *C*) had a rapid and dramatic effect on the distribution of gp74 which disappeared from the Golgi and was detected in numerous punctate structures scattered throughout the cytoplasm (Fig. 10, *D, H, L, F, J,* and *N*). In contrast, the resident proteins GMP_{c-1} (Fig. 10, *E, I, M*) and GMP_{t-1} (Fig. 10, *G, K, O*) remained in *cis*- and *trans*-Golgi elements and drifted with them upon their disruption. The results implicate microtubules in the cycling of gp74 which appeared to be unable to return to the slowly disintegrating Golgi complex. In this respect, it is interesting to note that pretreatment of cells with $[AlF_4]^-$ prevented the effects of nocodazol on both the redistribution of gp74 and the disruption of Golgi elements (Fig. 11) (see Discussion).

Discussion

Using a monoclonal antibody raised to rat liver membranes

we have identified a novel CGN membrane protein that cycles through the ER/IC. The antibody recognizes a glycoprotein of 74 kD (gp74) that has a polypeptide backbone of 34 kD.

The high content in N-linked complex carbohydrates, though unexpected, is not novel as proteins resident in the early Golgi (Yuan et al., 1987; Gonatas et al., 1989) and ER (Peter et al., 1992) have been reported to have complex carbohydrates. It is likely that proteins located in *cis* compartments acquire complex carbohydrates by virtue of their cycling through or escaping to the Golgi stacks where reside the enzymes involved in the processing of simple to complex carbohydrates (Kornfeld and Kornfeld, 1985; Pelham, 1991; Peter et al., 1992). This is consistent with the observations that decreasing but significant levels of gp74 are detected in the second and third cisternae of the Golgi stack (see Table I *B*).

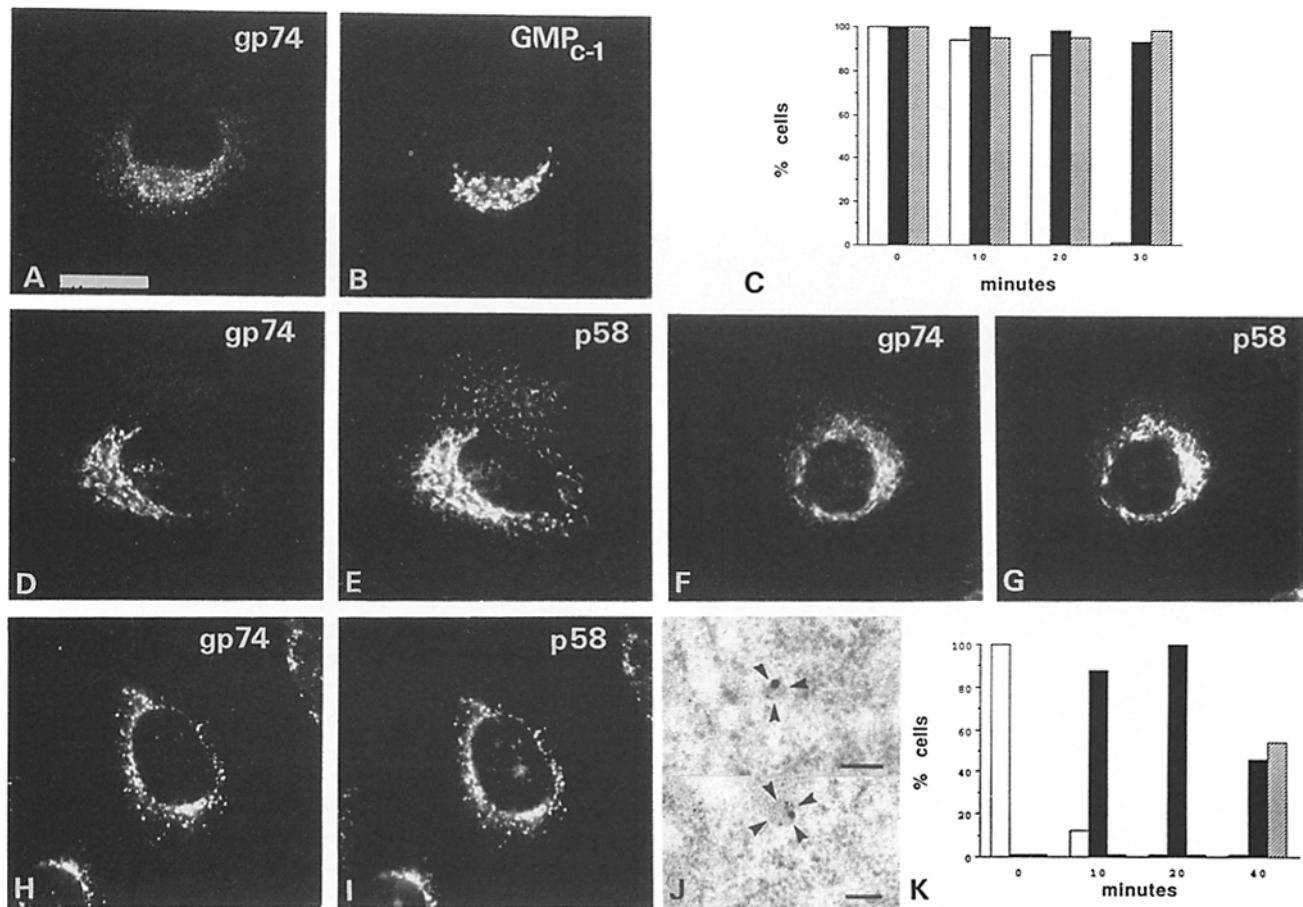Immunofluorescence microscopy studies show that the an-
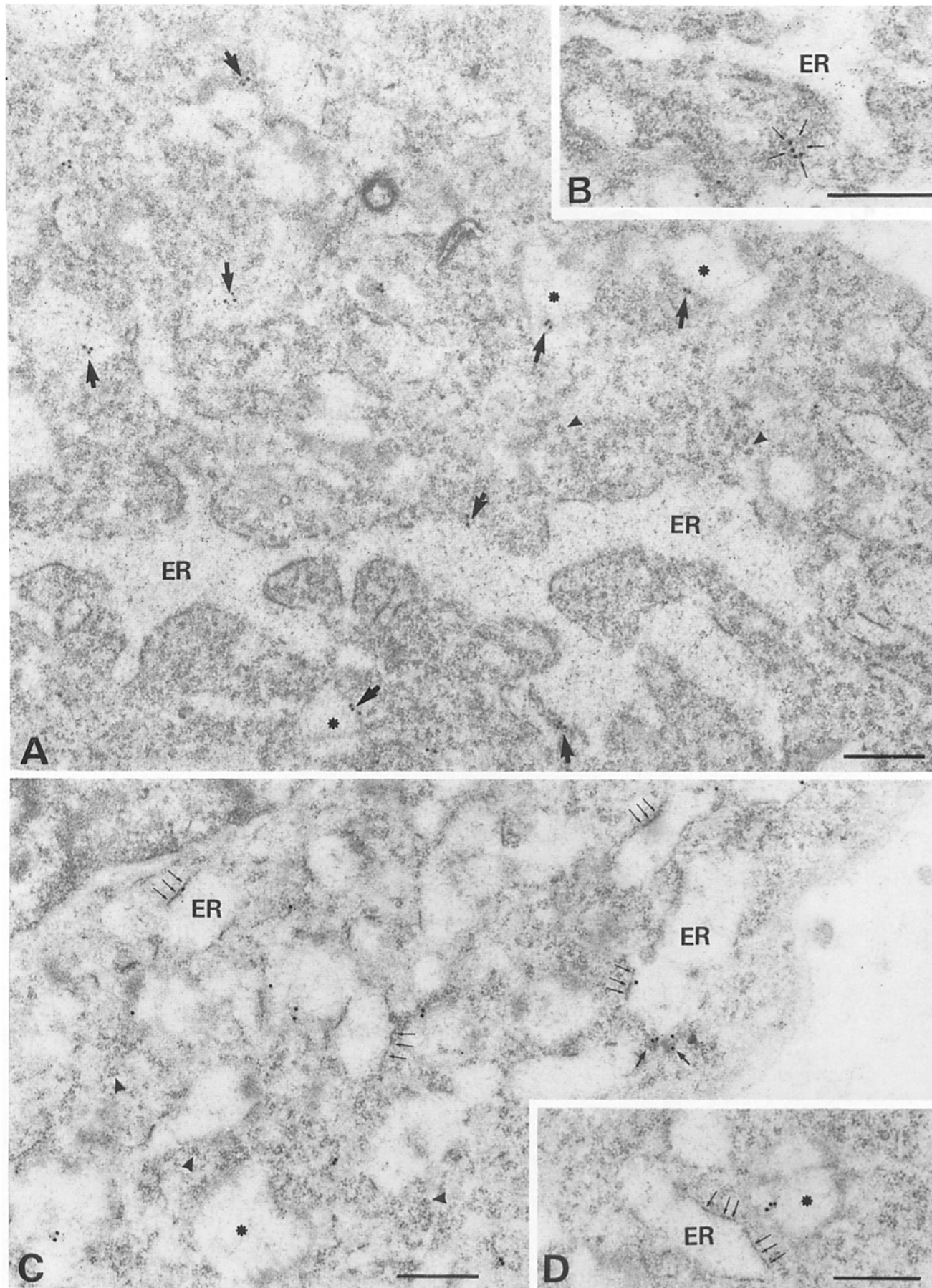
Figure 8. Redistribution of gp74 and p58 in response to [AlF$_4$]$^-$. NRK cells were treated for 30 min with 50 μM [AlF$_4$]$^-$ and double stained for gp74 (*A*, fluorescein channel) and GMP$_{c-1}$ (*B*, rhodamine channel). Note the relocation of gp74 to punctate structures clustered in the vicinity of the Golgi elements stained by GMP$_{c-1}$. (*C*) Percentage of cells that incubated with 50 μM [AlF$_4$]$^-$ for the indicated times and stained for gp74 (□), GMP$_{c-1}$ (■), or GMP$_{t-1}$ (▨) showed intact Golgi elements. Cells treated for 10 min (*D* and *E*), 20 min (*F* and *G*), or 40 min (*H* and *I*) with 50 μM [AlF$_4$]$^-$ and double stained for gp74 (*D*, *F*, and *H*, rhodamine channel) and p58 (*E*, *G*, and *I*, fluorescein channel). (*J*) EM of cells treated as in *H* and *I*; vesicles loaded with gp74 (gold 10 nm) and p58 (gold 15 nm). (*K*) Time course of the redistribution of p58 in cells treated with 50 μM [AlF$_4$]$^-$. Percentage of cells displaying preferential accumulation of p58 in gp74 negative (□) or positive punctate structures (▨) and in Golgi elements (■). Bars: (*A* and *B*) 15 μm; (*D–I*) 15 μm; (*J*) 100 nm.

tibody to gp74 strongly stains the same reticular structure visualized by antibodies to the Golgi resident membrane proteins GMP$_{c-1}$ and GMP$_{t-1}$ (Yuan et., 1987), indicating that the bulk of gp74 is in the Golgi complex. Studies on the distribution of gp74 within the Golgi complex, performed by immunogold EM, show that gp74 is located in a compartment opposite the *trans*-Golgi that contains sialylated proteins (Roth et al., 1985, 1986) and suggests that gp74 is expressed in the *cis*-Golgi. Its position *cis* to GMP$_{c-1}$, a marker of the *cis*/middle cisternae (see Table I) and codistribution with the Tn antigen, GalNacα-O-Ser/Thr, seen in glycoproteins that are not transported beyond the early *cis*-Golgi (Takahashi et al., 1988), suggests that gp74 is located

in the *cis*-most Golgi. This conclusion is also supported by the localization of gp74 in cisternae with the fenestrated morphology characteristic of the first Golgi cisterna (Rambourg et al., 1979) which is part of the CGN (Lindsey and Ellisman, 1985; Rambourg and Clermont, 1990; Mellman and Simons, 1992). Furthermore, the rapid redistribution of gp74 upon addition and removal of BFA is also consistent with its retention in the CGN (Alcalde et al., 1992).

In addition to the Golgi complex gp74 is also localized in numerous punctate structures which, though clustered in the vicinity of the Golgi, are not stained by *cis*- and *trans*-Golgi resident markers. At the EM level, the gp74 localized outside the Golgi complex is found as foci within the ER cisternae,

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Figure 7. Localization of gp74 in cells treated with BFA. Lowicryl sections of NRK cells incubated for 10 min with BFA and double stained for gp74 (15 nm gold) and PDI (10 nm gold) (*A* and *B*) or for gp74 (10 nm gold) and p58 (15 nm gold) (*C* and *D*). (*A*) Clusters of gp74 labeling the membranes of swollen ER cisternae loaded with PDI and of large pleomorphic vesicles lacking PDI and ribosomes (*) are visible. (*B*) Vesicle loaded with gp74 (the arrows mark the contour of the vesicle). (*C* and *D*) Labeling of ER membranes (*large arrows*) and of pleomorphic vesicles with membranes that lack ribosomes (*) by gp74 and p58. ER membranes decorated with ribosomes are marked with three small arrows and glycogen rosettes with arrowheads. Bars, 500 nm.
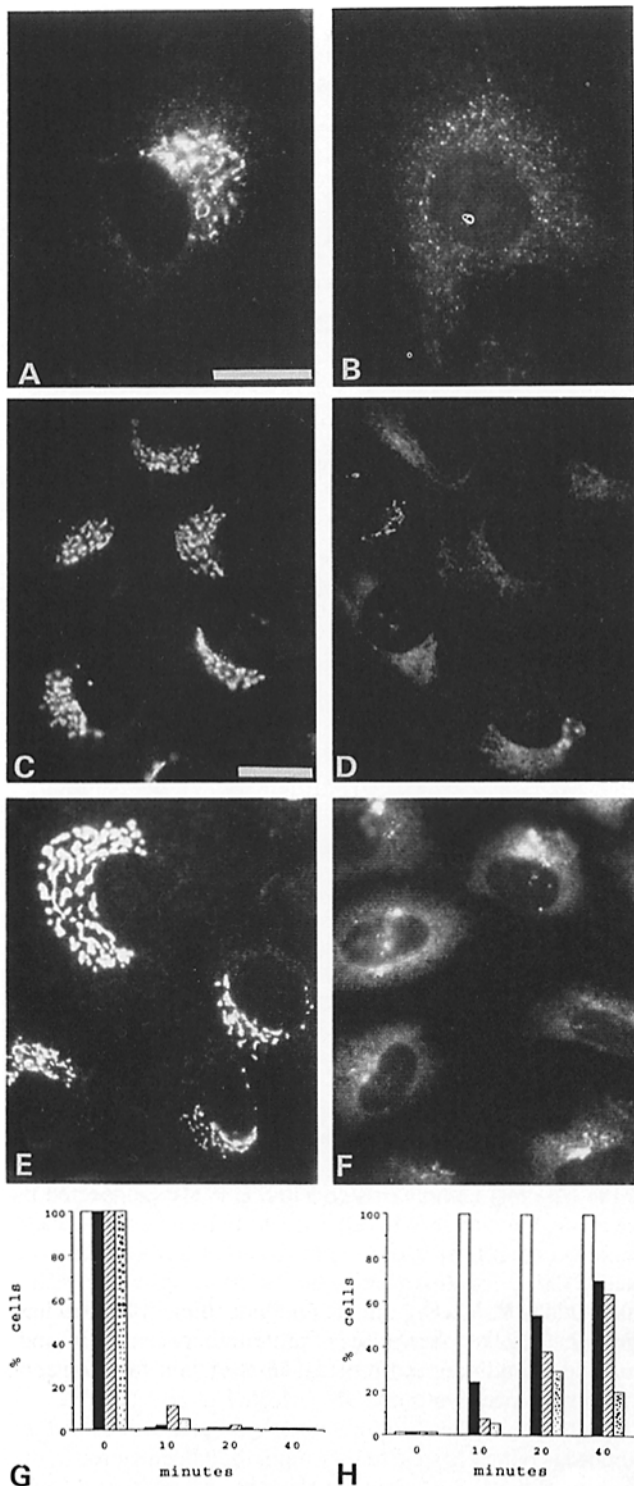
Figure 9. [AlF₄]⁻ does not retard the redistribution of gp74 in cells treated with BFA. NRK cells incubated in normal medium stained for gp74 (*A*), GMP$_{c-1}$ (*C*), or α-mannosidase II (*E*). Cells incubated for 10 min with 50 μM [AlF₄]⁻ before continuing the incubation for 10 min with 1 μg/ml BFA, stained for gp74 (*B*), GMP$_{c-1}$ (*D*), or α-mannosidase II (*F*). Percentage of cells that preincubated for 10 min with 50 μM [AlF₄]⁻ and then for the indicated times with 50 μM [AlF₄]⁻ and 1 μg/ml BFA, when stained for gp74 (□), GMP$_{c-1}$ (■), α-mannosidase II (▨), or GMP$_{t-1}$(▩) showed intact (*G*) or disassembled Golgi complexes (*H*). Bars: (*A–F*) 15 μm.

in smooth tubules, large pleomorphic vesicles with smooth surfaces and electrolucent lumens (378 nm) and small vesicles (60–75 nm). The CGN has been described as the part of the Golgi complex constituted by the first cisternae and an attached network of tubules (Lindsey and Ellisman, 1985; Rambourg and Clermont, 1990; Mellman and Simons, 1992), though the characterization and determination of the extension and connections of the tubular components of the CGN remains to be rigorously defined (Rothman and Orci, 1992; Mellman and Simons, 1992). In view of its localization in the *cis*-most Golgi and vesiculotubular structures and evidence that it cycles through the ER/IC (see below) it is likely that gp74 resides most of the time in the CGN. It is also important that the large pleomorphic and small vesicles that host gp74 frequently contain the itinerant IC protein p58 (Saraste and Svensson, 1991) and the KDEL-tagged PDI, proteins that also cycle between the ER/IC and the Golgi complex and suggests that these structures could be involved in the cycling of gp74 between the ER/IC and the CGN.

The comparison of the cellular distributions of gp74 and the IC p58 was particularly interesting as they both appear to be itinerant proteins. The observation that the majority of the gp74 and p58 molecules are segregated in cells incubated at 37°C, codistribute in the Golgi at 20°C and overlap in the IC at 15°C suggest that both proteins cycle between the ER/IC and CGN. The results suggest that at 20°C p58 can leave the IC and is retained in the Golgi, probably due to a slowdown of the transport from the Golgi to the IC. In contrast, at 15°C the two proteins appear to leave the Golgi complex and are retained in the IC by the blocking of the transport from the IC to the Golgi (Saraste et al., 1986; Kuismanen and Saraste, 1989; Schweizer et al., 1990; Saraste and Svensson, 1991).

The relation between the ER and the IC remains unclear. The IC has recently been renamed the *trans*-endoplasmic reticulum network (Hauri and Schweizer, 1992), a term that stresses the existing evidence of its morphological and functional connections with the ER and considers the IC as the sum of the transport outposts of the ER. In this respect it is unclear whether the tubular structures believed to constitute the IC (Schweizer et al., 1988; Saraste and Svensson, 1991) form a network separated or continuous with the ER cisternae. The ER foci that contain clusters of gp74 and p58 could correspond to the transport outposts of the ER, but it is not clear if they are part of the IC. The dramatic redistribution of gp74 and p58 into pleomorphic vesicles upon addition of BFA and the detection of gp74/p58 foci in the ER of these cells, suggests that gp74 was trapped in IC elements distinct from the ER foci, and that these may not be part of the IC. The relocation of gp74 to p58 positive vesicular structures in response to BFA might reflect the continuous access of this Golgi protein to the IC, a possibility that is supported by the observation that the resident protein GMP$_{c-1}$ is, in contrast, translocated to the ER (Alcalde et al., 1992). It is, however, unclear why the bulk of gp74 is retained in structures that contain p58. It has been suggested that BFA blocks the anterograde transport between the ER/IC and the Golgi (Lippincott-Schwartz et al., 1990a; Orci et al., 1991). However, since we do not observe any decrease in the staining of gp74/p58 positive vesicles on prolonged incubations with BFA, it is likely that the drug also blocks the transport from IC elements to the ER. If the retrograde transport is blocked,

the observation that the translocation of resident Golgi proteins to the ER continues in cells containing gp74 localized to vesicular structures (Alcalde et al., 1992) would support the possibility that proteins can be translocated directly from the Golgi to the ER, probably through tubules that emerge from the Golgi (Lippincott-Schwartz et al., 1990).

BFA has also been a useful tool in the study of the transport-related phenomenon of Golgi assembly, which upon removal of the drug has been shown to proceed in a cis to trans direction (Alcalde et al., 1992). The observation that the Golgi reticulum stained by gp74 assembles rapidly upon removal of the drug is again consistent with the retention of gp74 in the CGN. The differences between the rates of assembly of the Golgi elements containing gp74 (8–10 min) and GMP$_{c-1}$ (1 h; Alcalde et al., 1992) is consistent with EM data indicating that gp74 and GMP$_{c-1}$ are localized in different Golgi compartments (Table I A). The possibility that these differences are due to the asynchronous return of gp74 and GMP$_{c-1}$ to a Golgi pattern is less likely as the Golgi elements stained by gp74 are assembled within 8 to 10 min after removal of BFA and, within the same period of time, GMP$_{c-1}$ was released from the ER, even though the Golgi elements stained by GMP$_{c-1}$ were only assembled after 1 h.

The redistribution of gp74 to vesicles in [AlF$_4$]$^-$-treated cells is again in contrast with the absence of any effect on the distribution of GMP$_{c-1}$ and also supports the idea of gp74 is an itinerant protein. It is interesting that [AlF$_4$]$^-$ treatment causes the rapid disappearance of p58 from vesicle-like structures and accumulation in the Golgi complex but that, in a subsequent phase, the protein is relocated to gp74-positive vesicles. The early effect probably reflects the rapid consumption of uncoated vesicles and/or an increase in the coating of Golgi membranes resulting in the slowdown of vesicle budding and retention of the protein in the organelle. The accumulation of gp74 and p58 in vesicles after a lag of 20 min, is consistent with their loading into slow budding transport vesicles that are unable to lose their coats and fuse with the acceptor organelles (Melançon et al., 1987).

It has previously been reported that [AlF$_4$]$^-$ blocks the BFA induced translocation of Golgi proteins to the ER, and interferes with the disassembly of the Golgi complex, by securing the coats attached to Golgi membranes (Donaldson et al., 1991; Ktistakis et al., 1992). The fastening of coats to Golgi membranes could decrease the mobility of membrane proteins, increase the stability of the organelle, and result in the inhibition of the vesicle-mediated transport (Melançon et al., 1987; Stow et al., 1992). Such a mechanism would explain the slow translocation of GMP$_{c-1}$, GMP$_{t-1}$, and α-mannosidase to the ER in the cells pretreated with [AlF$_4$]$^-$ and the lack of effect of [AlF$_4$]$^-$ on the redistribution of gp74 to vesicle-like structures reflect the itinerant character of gp74 and its ability to escape from coated membranes that retain resident proteins with high efficiency.

Finally, the rapid dispersion of gp74 into punctate structures upon microtubule depolymerization is also distinct from the response of resident Golgi membrane proteins. The involvement of microtubule motors in vesicular transport (Vale and Goldstein, 1990; Vallee and Shpetner, 1990; Bliek and Meyerowitz, 1991) and the translocation of gp74 to disperse punctate structures suggest that the cycling of gp74 is disorganized in the absence of microtubules. The dispersion

is, however, also consistent with the possibility of a rapid disruption of the gp74 containing compartment as microtubules have also been implicated in maintaining the integrity and localization of the Golgi complex (Kupffer et al., 1982, 1983; Wehland et al., 1983a,b; Sandoval et al., 1984; Turner and Tartakoff, 1989; Kreis, 1990; Corthésy-Theulaz, 1992) and the gp74-stained reticular structure shows discontinuities that are in sharp contrast with the integrity of Golgi elements stained by cis- and tran-resident membrane proteins. Since previous results have shown that in cells treated with BFA the disassembly of the Golgi complex proceeds in a cis to trans direction (Alcalde et al., 1992), we speculate that microtubule depolymerization might cause the rapid disruption of a highly fragile CGN and initiate the collapse of the entire Golgi complex.

It is interesting that whereas [AlF$_4$]$^-$ promotes the slow redistribution of gp74 to vesicles in cells with intact microtubules it inhibits the rapid redistribution of the protein that follows microtubule depolymerization. The inhibitory effect could result from extensive coating and stabilization of the Golgi membranes that may inhibit vesicle budding and/or Golgi disruption in the absence of microtubules.
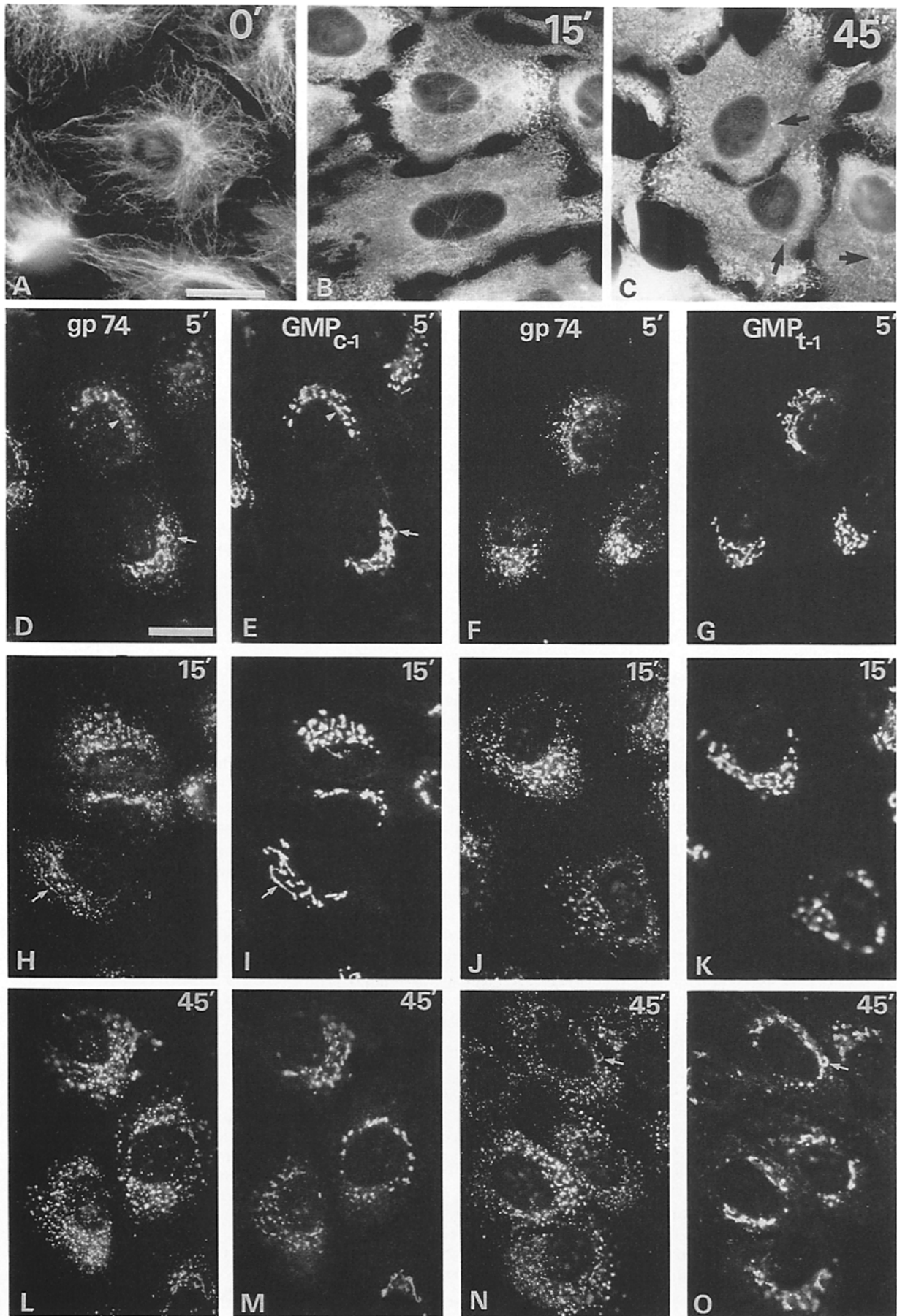
We do not know the function of gp74. Among the mammalian Golgi proteins reported in the literature gp74 shows a similar distribution and cycling behavior to the 23-kD KDEL receptor (Lewis and Pelham, 1992; Tang et al., 1993). The KDEL receptor retrieves luminal ER proteins from later stages of the secretory pathway and has been involved in the regulation of the retrograde flow (Lewis and Pelham, 1992). A quite distinct signal (KKXX or KXKXX) has been identified on the cytoplasmic tail of the adenovirus E3/19K and other resident ER membrane proteins, suggesting an equally specific receptor and the possibility that it would act to retrieve protein molecules that escape the ER (Jackson et al., 1990). The accumulation of gp74 in the CGN and its cycling through the ER/IC suggests that could be involved either in the retrieval of ER proteins or in regulating the retrograde flow. We are presently examining these possibilities. As the scarcity of accepted marker proteins is a major limitation to studies on the structure and function of the cis-most Golgi (i.e., CGN), new markers like gp74, used in conjunction with other markers of the IC should enable better definition of the boundaries between the CGN and IC and characterize their structures and dynamics. It is likely that from such studies and from the understanding of the mechanisms that regulate the cycling of proteins between the IC and Golgi complex a more complete picture of their function and relationships will emerge.
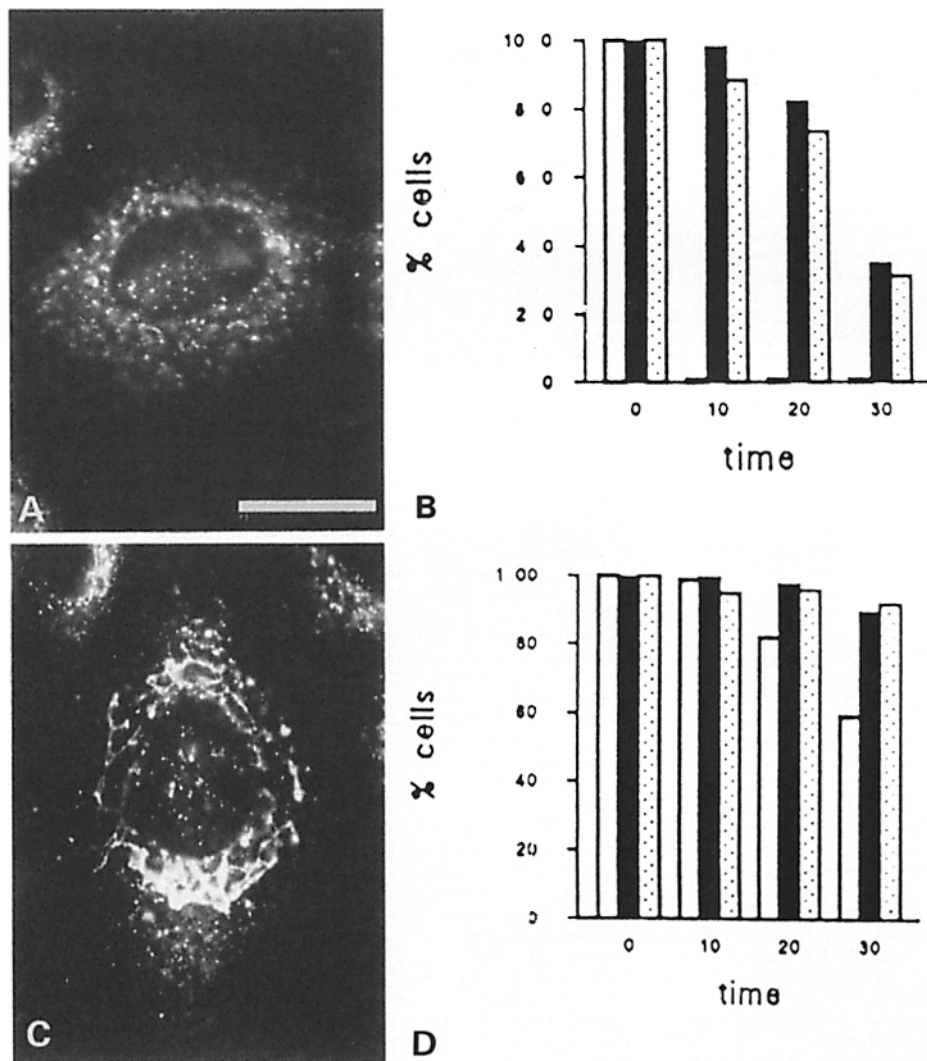
Figure 11. [AlF₄]⁻ delays the redistribution of gp74 and the disruption of the Golgi complex in cells treated with nocodazol. (*A*) NRK cells incubated for 30 min with 20 μM nocodazol, stained for gp74. (*C*) NRK cells incubated for 10 min with 50 μM [AlF₄]⁻ before continuing the incubation for 30 min with 20 μM nocodazol, stained for gp74. (*B* and *D*) Percentage of cells that incubated as in *A* (*B*) or *C* (*D*) and stained for gp74 (□), GMP$_{c-1}$ (■), or GMP$_{t-1}$ (▨) displayed intact Golgi complexes. Bars, 15 μM.

References

Alcalde, J., P. Bonay, A. Roa, S. Vilaró, and I. V. Sandoval. 1992. Assembly and disassembly of the Golgi complex: two processes arranged in a cis-trans direction. *J. Cell Biol.* 116:69–83.

Barriocanal, J. G., J. S. Bonifacino, L. Yuan, and I. V. Sandoval. 1986. Biosynthesis, glycosylation, movement through the Golgi system, and transport to lysosomes by a N-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. *J. Biol. Chem.* 261:16755–16763.

Beckers, C. J. M., and W. E. Balch. 1989. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J. Cell Biol.* 108:1245–1256.

Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell.* 50:523–554.

Bliek, A. M., and E. M. Meyerowitz. 1991. Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. *Nature (Lond.).* 351:411–414.

Bonatti, S., G. Miglaccio, and K. Simons. 1989. Palmitoylation of viral membrane glycoproteins takes place after exit from the endoplasmic reticulum. *J. Biol. Chem.* 264:12590–12595.

Bonifacino, J. S., P. Perez, R. D. Klausner, and I. V. Sandoval. 1985. Study of the transit of an integral membrane protein from secretory granules through the plasma membrane of secreting rat basophilic leukaemia cells using a specific monoclonal antibody. *J. Cell Biol.* 102:516–522.

Bordier, C. 1981. Phase separation of integral membrane proteins in triton X-114 solution. *J. Biol. Chem.* 256:1604–1607.

Carlemaln, E., R. M. Garavito, and W. Villiger. 1982. Resin development for electron microscopy and an analysis of embedding at low temperature. *J. Microsc.* 126:123–143.

Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell.* 62:317–329.

Corthéshy-Theulaz, I., A. Pauloin, and S. R. Pfeffer. 1992. Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. *J. Cell Biol.* 118:1333–1345.

Dean, N., and H. R. B. Pelham. 1990. Recycling of proteins from the Golgi compartment to the ER in yeast. *J. Cell Biol.* 111:369–377.

Figure 10. Microtubule depolymerization causes the rapid redistribution of gp74 to punctate structures and the slow disruption of the Golgi complex. NRK cells incubated without (*A*) or with 20 μM nocodazol for 15 (*B*) or 45 min (*C*, centrosomes are marked with *arrows*) stained for tubulin. Cells incubated for 5 min (*D–G*), 15 min (*H–K*), or 45 min (*L–O*) with 20 μM nocodazol and double stained for gp74 (*D*, *H*, and *L*, fluorescein channel) and GMP$_{c-1}$ (*E*, *I*, and *M*, rhodamine channel), or for gp74 (*F*, *J*, and *N*, fluorescein channel), and GMP$_{t-1}$ (*G*, *K*, and *O*, rhodamine channel). Note the rapid redistribution of gp74 and the slow disruption of the Golgi elements stained by GMP$_{c-1}$ and GMP$_{t-1}$. Arrows mark Golgi elements that uniformly labeled by the antibodies to GMP$_{c-1}$ and GMP$_{t-1}$ show discontinuities when labeled with the antibody to gp74. Bars, 15 μm.

Doms, R. W., G. Russ, and J. W. Yewdell. 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* 109:61-72.

Donaldson, J. G., J. Lippincott-Schwartz, and R. D. Klausner. 1991. Guanine nucleotides modulate the effects of brefeldin A in semipermeable cells: regulation of the association of a 110kD peripheral membrane protein with the Golgi apparatus. *J. Cell Biol.* 112:579-588.

Dunphy, W. G., and J. E. Rothman. 1985. Compartmental organization of the Golgi stack. *Cell.* 42:13-21.

Farquhar, M. G., and G. E. Palade. 1981. The Golgi apparatus complex-(1954-1981)-from artifact to center stage. *J. Cell Biol.* 91:77s-103s.

Frens, G. 1973. Preparation of gold dispersions of varying particle size: controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. *Nature: Phys. Sci.* 241:20-22.

Gonatas, J. O., S. G. E. Mezitis, A. Stieber, B. Fleichser, and N. K. Gonatas. 1989. A novel sialoglycoprotein of the medial cisternae of the Golgi apparatus. *J. Biol. Chem.* 254:646-653.

Gonzalez, M. E., F. Martinez Abarca, and L. Carrasco. 1990. Flavonoids: potent inhibitors of poliovirus RNA synthesis. *Antiviral Chem. Chemother.* 1:203-209.

Griffiths, G., and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438-442.

Hauri, H. P., and A. Schweizer. 1992. The endoplasmic reticulum-Golgi intermediate compartment. *Curr. Opin. Cell Biol.* 4:600-609.

Jackson, M. R., T. Nilsson, and P. A. Peterson. 1990. Identification of the consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3153-3162.

Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576-582.

Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine linked oligosaccharides. *Annu. Rev. Biochem.* 54:631-664.

Kreis, T. 1990. Role of microtubules in the organization of the Golgi apparatus. *Cell Motil. Cytoskeleton.* 15:67-70.

Ktistakis, N. T., M. E. Linder, and M. R. Roth. 1992. Action of brefeldin A blocked by activation of a pertussis-toxin-sensitive protein. *Nature (Lond.).* 356:344-346.

Kuismanen, E., and J. Saraste. 1989. Low temperature-induced transport blocks as tools to manipulate membrane traffic. *Meth. Cell Biol.* 32:257-274.

Kupffer, A., D. Louvard, and J. singer. 1982. Polarization of the Golgi apparatus and the microtubule organizing center in cultured fibroblasts at the edge of an experimental wound. *Proc. Natl. Acad. Sci. USA.* 79:2603-2607.

Kupffer, A., G. Dennert, and S. J. Singer. 1983. Polarization of the Golgi apparatus and microtubule organizing center within clone natural killer cells bound to their targets. *Proc. Natl. Acad. Sci. USA.* 80:7224-7228.

Lahtinen, U., B. Dahllof, and J. Saraste. 1992. Characterization of a 58 kD protein in pancreatic exocrine cells. *J. Cell Sci.* 103:321-333.

Lambert, N., and R. D. Freedman. 1985. The latency of rat liver microsomal protein-disulphide-isomerase. *Biochem. J.* 228:635-645.

Lewis, M. J., and H. R. B. Pelham. 1992. Ligand induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell.* 68:353-364.

Lindsey, J. D., and M. H. Ellisman. 1985. The neuronal endomembrane system. II. The multiple forms of the Golgi apparatus cis element. *J. Neurosci.* 5:3124-3134.

Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the endoplasmic reticulum in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell.* 56:801-813.

Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H. P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an Er recycling pathway. *Cell.* 60:821-836.

Malhotra, V., T. Serafini, L. Orci, J. C. Sheperd, and J. E. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell.* 58:329-336.

Melançon, P., B. S. Glick, V. Malhotra, P. J. Weidman, T. Serafini, M. L. Gleason, L. Orci, and J. E. Rothman. 1987. Involvement of GTP binding "G" proteins in transport through the Golgi stack. *Cell.* 51:1053-1062.

Mellman, I., and K. Simons. 1992. The Golgi complex: in vitro veritas? *Cell.* 68:829-840.

Moremen, K., and O. Touster. 1985. Biosynthesis and modification of Golgi mannosidase II in HeLa and 3T3 cells. *J. Biol. Chem.* 260:6654-6662.

Orci, L., B. S. Glick, and J. E. Rothman. 1986. A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell.* 46:171-184.

Orci, L., V. Malhotra, M. Amherdt, T. Serafini, and J. E. Rothman. 1989. Dissection of a single round of vesicular transport: sequential intermediates for intercisternae movement in the Golgi stack. *Cell.* 56:357-368.

Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J. Donaldson, J. Lippincott-Schwartz, R. D. Klausner, and J. E. Rothman. 1991. Brefeldin A a drug that blocks secretion prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell.* 64:1183-1195.

Palade, G. E. 1975. Intracellular aspects of the process of protein sorting. *Science (Wash. D.C.).* 189:347-358.

Pascual, D. W., and L. W. Clem. 1992. Low temperature pepsin proteolysis. An effective method for mouse IgM (Fab')₂ fragments production. *J. Immunol. Methods.* 146:249-255.

Pelham, H. R. B. 1988. Evidence that luminal ER Proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913-918.

Pelham, H. R. B. 1989. Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:1-23.

Pelham, H. R. B. 1991. Recycling of proteins between the endoplasmic reticulum and Golgi complex. *Curr. Opin. Cell Biol.* 3:585-591.

Peter, F., P. van Nguyen, and H. D. Soeling. 1992. Different sorting of proteins in rat liver. *J. Biol. Chem.* 267:10631-10637.

Peter, F., H. Plutner, H. Zhu, T. E. Kreis, and W. E. Balch. 1993. β-COP is essential for transport of protein from the endoplasmic reticulum to the Golgi in vitro. *J. Cell Biol.* 122:1155-1167.

Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56:829-852.

Plutner, H., A. D. Cox, S. Pind, R. Kosravi-Far, J. R. Bourne, R. Schwanniger, C. J. Der, and W. E. Balch. 1991. Rab₁ᵦ regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. *J. Cell Biol.* 115:31-43.

Plutner, H., H. W. Davidson, J. Saraste, and W. E. Balch. 1992. Morphological analysis of protein transport from the ER to Golgi membranes in digitonin-permeabilized cells: role of the P58 containing compartment. *J. Cell Biol.* 119:1097-1116.

Rambourg, A., Y. Clermont, and L. Hermo. 1979. Three dimensional architecture of the Golgi apparatus in Sertoli cells of the rat. *Am. J. Anat.* 154:455-476.

Rambourg, A., and Y. Clermont. 1990. Three dimensional electron microscopy structure of the Golgi apparatus. *Eur. J. Cell Biol.* 51:189-200.

Reaves, B., and G. Banting. 1992. Perturbation of the morphology of the trans-Golgi network following brefeldin A treatment: redistribution of a TGN specific integral membrane protein, TGN38. *J. Cell Biol.* 116:85-94.

Rizzolo, L. J., and R. Kornfeld. 1988. Posttranslational protein modification in the endoplasmic reticulum. Demonstration of fatty acylase and deoxymannojirimycin-sensitive alpha-mannosidase activities. *J. Biol. Chem.* 263:9520-9525.

Roth, J. 1982. The Protein A-gold (pAg) technique: a qualitative and quantitative approach for antigen localization in thin sections. *In* Techniques in immunocytochemistry. Vol. 1. G. R. Bullock and P. Petrusz), editors. Academic Press, London, 107-133.

Roth, J., J. M. Lucocq, and P. Charest. 1984. Light and electron microscopy demonstration of sialic acid residues with the lectin from Limax flavus: a cytochemical affinity technique with the use of fetuin-gold complexes. *J. Histochem. Cytochem.* 32:1167-1176.

Roth, J., D. J. Taatjes, J. Lucocq, J. C. Weinstein, and P. Poulson. 1985. Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. *Cell.* 43:287-295.

Roth, J., D. J. Taatjes, J. Weinstein, J. C. Paulson, P. Greenwell, and W. M. Watkins. 1986. Differential subcompartmentation of terminal glycosylation in the Golgi apparatus of intestinal absorptive and goblet cells. *J. Biol. Chem.* 261:14307-14312.

Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature (Lond.).* 355:409-415.

Sandoval, I. V., J. S. Bonifacino, R. D. Klausner, M. Henkart, and J. Wehland. 1984. Role of microtubules in the organization and localization of the Golgi apparatus. *J. Cell Biol.* 99:113s-118s.

Saraste, J., and K. Svensson. 1991. Distribution of the intermediate elements operating in the ER to Golgi transport. *J. Cell Sci.* 100:415-430.

Saraste, J., M. G. Farquhar, and G. E. Palade. 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.

Saraste, J., G. E. Palade, and M. G. Farquhar. 1987. Antibodies to rat pancreas subfractions: identification of a 58 kD cis-Golgi protein. *J. Cell Biol.* 105:2021-2030.

Schweizer, A., J. Fransen, T. Bachi, L. Ginser, and H. P. Hauri. 1988. Identification by a monoclonal antibody of a 53 kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. *J. Cell Biol.* 107:1643-1653.

Schweizer, A., J. Fransen, K. Matter, T. E. Kreis, L. Ginsel, and H. P. Hauri. 1990. Identification of an intermediate compartment involved in protein transport from ER to Golgi apparatus. *Eur. J. Cell Biol.* 53:185-196.

Schweizer, A., G. Griffiths, T. Bachi, and H. P. Hauri. 1991. Organization of the exocytic pathway from ER to Golgi. *J. Cell Biol. Abstr.* 374:65a.

Serafini, T., G. Stenbeck, A. Brecht, F. Lottspeich, L. Orci, J. E. Rothman, and F. T. Wieland. 1991. Identification of β-COP, a subunit of the coat of Golgi-derived (non-clathrin) coated vesicles, as a homologue of the clathrin-coated vesicle coat protein β-adaptin. *Nature (Lond.).* 349:215-220.

Slot, J. W., and H. J. Geuze. 1985. A new method of preparing gold probes for multiple-labelling cytochemistry. *Eur. J. Cell Biol.* 38:87-93.

Stow, J. L., J. B. de Almeida, N. Narula, E. J. Holtzman, L. Ercolani, and D. A. Ausiello. 1992. A heterotrimeric G protein, Gα₁₋₃, on Golgi membranes regulates the secretion of heparan sulfate proteoglycan in LLC-PK₁ epithelial cells. *J. Cell Biol.* 114:1113-1124.

Tae, H., and I. Ji. 1982. Macromolecular photoaffinity labeling with radioactive photoactivable heterobifunctional reagents. *Anal. Biochem.* 121:286-289.

Takahashi, H. K., R. Metoki, and S. I. Hakomori. 1988. Immunoglobulin G3 monoclonal antibody directs to Tn antigen (tumor-associated α-N-acetylgalactosaminyl epitope) that does not cross-react with blood group A antigen. *Cancer Res.* 48:4361-4367.

Tang, B. L., S. H. Wong, X. L. Qi, S. H. Low, and W. Hong. 1993. Molecular cloning, characterization, subcellular localization and dynamics of p23, the mammalian KDEL receptor. *J. Cell Biol.* 120:325-338.

Tooze, S., J. Tooze, and G. Warren. 1988. Site of addition of N-acetylgalactosamine to the E_1 glycoprotein of mouse hepatitis virus-A59. *J. Cell Biol.* 106:1475-1487.

Turner, J. R., and A. M. Tartakoff. 1989. The response of the Golgi complex to microtubule alterations: the roles of metabolic energy and membrane traffic in Golgi complex organization. *J. Cell Biol.* 109:2081-2088.

Ulmer, J. B., and G. E. Palade. 1989. Targeting and processing of glycophorins in murine erythroleukemia cells: use of Brefeldin A as a perturbant of intracellular traffic. *Proc. Natl. Acad. Sci. USA.* 86:6992-6996.

Vale, R. D., and L. S. B. Goldstein. 1990. One motor, many tails: An expanding repertoire of force-generating enzymes. *Cell.* 60:883-885.

Vallee, R. B., and H. S. Shpetner. 1990. Motor proteins of cytoplasmic microtubules. *Annu. Rev. Biochem.* 59:909-932.

Wehland, J., and I. V. Sandoval. 1983a. Cells infected with guanosine 5' (a, b methylene) triphosphate, and a β-non-hydrolysable analog of GTP show anomalous patterns of tubulin polymerization affecting cell translocation, intracellular movement and the organization of Golgi elements. *Proc. Natl. Acad. Sci. USA.* 80:1938-1941.

Wehland, J., M. Henkart, R. D. Klausner, and I. V. Sandoval. 1983b. Role of microtubules in the distribution of the Golgi apparatus: effect of taxol and microinjected anti-α-tubulin antibodies. *Proc. Natl. Acad. Sci. USA.* 80:4286-4290.

Yuan, L., J. G. Barriocanal, J. S. Bonifacino, and I. V. Sandoval. 1987. Two integral membrane proteins located in the cis-middle and transport of the Golgi system acquire sialylated N-linked carbohydrates and display different turnovers and sensitivity to cAMP-dependent phosphorylation. *J. Cell Biol.* 105:215-227.