A Peripheral Protein Associated with the *cis*-Golgi Network Redistributes in the Intermediate Compartment upon Brefeldin A Treatment

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Abstract. Human autoantibodies offer unique tools for the study of cellular constituents since they usually recognize highly conserved components, the most difficult to detect due to their low immunogenicity. The serum from a patient with Sjögren's syndrome (RM serum) showing a very high reactivity to the Golgi complex has been shown to immunoprecipitate and to immunodetect by Western blotting experiments a protein of mol wt 210,000 (p210) that was shown to be peripheral and cytoplasmically disposed. A close examination of the p210 labeling revealed some differences with Golgi markers: RM serum staining was slightly more extensive than several Golgi markers and showed a discontinuous or granular appearance. Nocodazole induced a specific and early segregation of many p210-associated vesicles or tubules from Golgi apparatus. Upon brefeldin A treatment, p210 did not redistribute in the ER as did other Golgi proteins. In contrast, it exhibited a vesicular pattern reminiscent to that displayed by proteins residing in the intermediate compartment. Double staining immunofluorescence using the RM serum and the marker of the intermediate compartment, p58, revealed segregation of both proteins in control conditions but colocalization in BFA-treated cells. We have further demonstrated by combining different drug treatments that p210-containing elements in brefeldin A-treated cells belong indeed to the intermediate compartment. Experiments on brefeldin A recovery suggested that these p210 elements might play a role in reformation and repositioning of the Golgi apparatus. Ultrastructural localization performed by immunoperoxidase staining allowed us to establish that p210 interacted with the external side of an abundant tubulo-vesicular system on the *cis* side of the Golgi complex which extended to connecting structures and vesicles between saccules or stacks of cisternae. p210 appears to be a novel protein residing in the *cis*-Golgi network that may cycle between the Golgi apparatus and the intermediate compartment.

The Golgi apparatus (GA)[1] is a dynamic membranous network which plays a key role in processing, maturation, and sorting of the newly synthesized proteins destined to specific membrane domains, or to secretion, and in recycling of receptors involved in endocytosis. Whereas ER is usually extended throughout the cytoplasm, the Golgi complex appears centrally located in close proximity to the microtubule-organizing center (Farquhar and Palade, 1981; Kupfer et al., 1982). This pericentrosomal organization that is generated and maintained by microtubules (Wehland and Willingham, 1983; Wehland et al., 1983; Rogalski and Singer, 1984; Sandoval et al., 1984) enables the Golgi complex to receive and to direct both exocytic and endocytic material that traffic through it.

Functional and morphological studies have shown a polarized organization of the Golgi complex. A three compartment subdivision of the Golgi complex is thought to be required to accomplish all of its functions: the *cis*-Golgi network (CGN) next to the ER, where newly synthesized material is received and sorted, the *medial* Golgi (containing the *cis*, *medial*, and *trans* cisternae) where glycosylation and trimming of carbohydrate moieties takes place, and the *trans*-Golgi network (TGN) that mediates the sorting and exit of material from the GA before a final vesicular trans-

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1. *Abbreviations used in this paper*: ARF, ADP-ribosylation factor; CGN, *cis*-Golgi network; TGN, *trans*-Golgi network; BFA, brefeldin A; GA, Golgi apparatus; GalTf, antigalactosyl transferase; SLO, streptolysin O.

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port step to the terminal destination (Mellman and Simons, 1992).

Transport events through the GA are thought to occur by movement of carrier vesicles (Palade, 1975). Nonclathrin-coated vesicles have been proposed to mediate transport from ER to the Golgi complex and between the different Golgi compartments (Orci et al., 1986; Malhotra et al., 1989; Serafini et al., 1991*a*). The coat of these vesicles comprises a set of four proteins named COPs (α, β, γ and δ-COPs) and probably two additional subunits of 20 and 36 kD (Serafini et al., 1991*a*). All of these proteins exist in a cytosolic complex, the coatomer, that may represent an unassembled precursor of the coat (Waters et al., 1991). In addition ADP-ribosylation factor (ARF), a small GTP-binding protein, has been identified as a component of the Golgi-derived coated vesicles (Serafini et al., 1991*b*). Both the coatomer and ARF associate reversibly to Golgi membranes (Donaldson et al., 1991*a*; Serafini et al., 1991*b*) and dynamics of this cycle is now well established (Donaldson et al., 1992*a*). Recently, a Golgi membrane enzyme has been identified which might determine the localization of membrane-bound ARF–GTP and hence the sites for vesicle budding (Donaldson et al., 1992*b*; Helms and Rothman, 1992). Other small GTP-binding proteins of the rab family have also been found in association with specific vesicles and organelles and are thought to have a crucial role in the targeting of vesicles to, and their fusion with, the appropriate acceptor organelles (Bourne, 1988; Goud and McCaffrey, 1991; Pfeffer, 1992). For many of them however, more conclusive information is still required to decide whether their localization is restricted to a single pair of donor–acceptor compartments (see Antony et al., 1992). Heterotrimeric G proteins have also been reported to be part of the transport machinery (Ercolani et al., 1990; Barr et al., 1991; Donaldson et al., 1991*b*; Stow et al., 1991; Ktistakis et al., 1992).

Various drugs proved to be useful to elucidate the dynamic equilibrium on which Golgi membrane organization is based. The fungal metabolite brefeldin A (BFA) had been shown to inhibit protein secretion and dramatically disintegrates the Golgi apparatus, resulting in the distribution of Golgi membranes (lipids and proteins) in the ER by a mechanism that requires energy and microtubules. Uncoated tubules emanating from the Golgi complex are observed rapidly after addition of BFA to cells suggesting that they are the structural intermediates that carry the Golgi membranes to the ER (for review see Klausner et al., 1992). The immediate effect of BFA is to prevent the assembly of COPs-coated vesicles (Orci et al., 1991) probably by interfering with the association of ARF and consequently of the coatomer complex with Golgi membranes (Donaldson et al., 1992*a*). Thus BFA would block anterograde traffic between ER and GA without affecting retrograde transport from GA to ER. Morphology and functioning of other organelles of the secretory pathway such as TGN or the endosomal system are also perturbed by BFA (Klausner et al., 1992). BFA-like phenotypes have been described in two different situations: overexpression of a human ERD-2–like protein (Hsu et al., 1992) and in a mutant CHO cell line (Zuber et al. 1991). All of these data indicate that the Golgi apparatus exists as a steady state structure and that the maintenance of its integrity requires a rigorous control of the balance between anterograde and retrograde traffic from ER up to terminal GA.

In response to BFA treatment, three different behaviors of Golgi proteins have been reported. Most of the Golgi proteins such as mannosidase II and IA (Lippincott-Schwartz et al., 1989), galactosyl-transferase (Lippincott-Schwartz et al., 1990), the proteins p58, p54, and p86 (Donaldson et al., 1990), and $GMP_{c-1,2}$, MG 160, and $GMP_{t-1,2}$, (Alcalde et al., 1992) redistribute to ER. β-COP (Donaldson et al., 1990), ARF (Donaldson et al., 1991*a*), and a p200 (Narula et al., 1992) dissociate from Golgi membranes to cytosol. Finally p58 (Saraste and Svensson, 1991) and p53 (Lippincott-Schwartz et al., 1990), two proteins which reside in the intermediate compartment, do not modify their localization since intermediate compartment preserves its identity in the presence of BFA.

Despite this major progress in understanding the function and organization of the Golgi complex, many Golgi membrane proteins remain to be discovered or characterized. Recently, a new immunological approach has been used in order to identify highly conserved Golgi components which have shown to be the most difficult to detect using classical strategies, namely the screening of sera libraries from patients with systemic autoimmune diseases (Kooy et al., 1992). Human autoantibodies offer unique tools for the study of cellular components since they recognize highly conserved and functionally important molecules. In addition, the autoantibodies usually react with the active sites of the antigen and therefore they are often capable of inhibiting its functional activity (Tan, 1991). By using this approach, we have selected a serum from a patient with Sjögren's syndrome containing high titer autoantibodies to the GA (Rios, R. M., M. C. Boissier, J. C. Homberg, and M. Bornens, manuscript submitted for publication). It recognizes a novel Golgi protein of 210 kD in a wide variety of cells and organisms. Biochemical and EM investigations indicate that p210 is a peripheral protein whose localization is restricted to the *cis* side of the Golgi complex and seems to correspond to the CGN. Interestingly, p210 displays an unusual behavior, compared to bona fide Golgi markers, when cells are treated with nocodazole or with BFA. In experiments using BFA, unlike most Golgi proteins, p210 does not redistribute to ER but to the intermediate compartment. These results suggest that certain Golgi proteins might be excluded from the pathway that redistributes Golgi membrane proteins to the ER and that the time course of Golgi reconstitution after BFA treatment occurs by sequential events, probably involving a subset of specific proteins.

Materials and Methods

Cell Culture

The KE37 cell line of T lymphoblastic origin was grown in RPMI 1640 medium containing 7% fetal calf serum. HeLa cells were maintained in DME supplemented with 10% fetal calf serum. Normal muscles were obtained from patients undergoing surgical operations. The biopsy specimens were collected in a sterile container containing culture medium and stored at 4°C. The specimens were dissociated as previously described (Tassin et al., 1985*a*). 2 mM Glutamine, 50 U/ml penicillin, and 50 μg/m streptomycin were included in all culture media.

Antibodies

Serum of the patient R.M. suffering from Sjögren's syndrome (see Rodriguez et al., 1982 and Blascheck et al., 1988) was aliquoted, sodium azide added, and then stored at −70°C. IgG fraction (10 mg/ml final concentration) was purified from whole serum on protein A-Sepharose columns and

stored in 50% glycerol at −70°C. CTR 433 was obtained from a library of monoclonal antibodies against centrosomes isolated from human lymphoblasts and has been previously characterized as a marker of the *medial* compartment of the GA (Jasmin et al., 1989). Affinity-purified antigalactosyl transferase (GalTf) antibody was kindly provided by Dr. Eric Berger (Institute of Physiology, Zurich, Switzerland). Affinity-purified anti-p58 polyclonal antibody (Saraste et al., 1987) was obtained from Dr. Jaako Saraste (Ludwig Institute of Cancer Research, Stockholm, Sweden). A specific antiendoplasmic reticulum antibody (Louvard et al., 1982) was the kind gift of Dr. D. Louvard (Institut Pasteur, Paris, France). Anti-β-tubulin antibody was purchased from Amersham Corp. (Arlington Heights, IL).

Specific antibodies from RM serum were affinity purified on nitrocellulose strips using immunoreactive proteins from different fractions, according to Krohne et al. (1982).

Immunofluorescence Microscopy

Cells were grown on culture-treated slides for 2 d before an experiment. Cells were rinsed twice with PBS and incubated in methanol at −20°C for 6 min to simultaneously fix and permeabilize the cells. After methanol treatment, cells were rinsed with PBS containing 0.1% Tween 20 (PBST). Primary antibodies diluted in PBST containing 3% BSA were added for 1 h at room temperature. The cells were then rinsed with PBST three times to wash away unbound primary antibodies. The same incubations and washing procedures were used for fluorescein- or rhodamine-labeled secondary antibodies. The cells were finally dehydrated, mounted in citifluor, examined, and photographed with a Zeiss Axiophot microscope. For the visualization of p58, the cells were fixed for 15 min at room temperature with 3% paraformaldehyde in 0.1% phosphate buffer, pH 7.4, and thereafter permeabilized for 15 min in PBS containing 0.1% saponin. After a rinse, the cells were incubated with 50 mM NH_4Cl to inactivate free aldehyde groups and then processed as described above.

Drug Treatments

BFA was purchased from Epicentre Technologies (Madison, WI) or obtained from Sandoz Ltd. (Basel, Switzerland). It was stored at −20°C as a stock solution of 5 mg/ml in methanol. Nocodazole was obtained from Sigma Chem. Co. (St. Louis, MO) and stored as a 5-mM stock solution in DMSO at −20°C. Immediately prior to use, solutions were prepared in culture medium. Cells were incubated at 37°C with 10 μM nocodazole or 1-5 μg/ml BFA for various periods up to 2 h. The calcium ionophore A23187 (Sigma Chem. Co.) was administered to cells in culture medium at 5 μM final concentration. Cells were incubated with A23187 for 4 h at 37°C before fixation.

Metabolic Labeling and Immunoprecipitation

Subconfluent monolayers of HeLa cells were labeled with 100–200 μCi/ml of [^{35}S]methionine/cysteine (Trans-Label; ICN Biomedicals, Inc., Costa Mesa, CA) for 4 h in DMEM supplemented with 5% FCS. Cells were washed with ice-cold PBS and incubated with 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100) containing 1/1,000 CLAP (10 μg/ml in DMSO of each chymostatin, leucopeptin, antipain, and pepstatin), 1 mM PMSF, and 1 μg/ml aprotinin for 30 min in ice with rocking. The cell lysate was collected and clarified by centrifugation at top speed in a microfuge for 5 min at 4°C. 50 μl of a 50% solution of protein A-Sepharose beads in lysis buffer were incubated with 1 or 2 μl of either autoimmune serum or normal human serum for 2 h in ice with rocking, washed, collected by centrifugation, and added to 50 μl of labeled samples. The mixture was rotated for 2 h at 4°C, then the protein A-Sepharose beads were collected and washed 5 times in lysis buffer followed by two washes in 50 mM Tris-HCl, pH 8.0. Proteins were released from the beads by boiling in SDS-PAGE sample buffer.

Subcellular Fractionation

Fractions enriched in Golgi vesicles were isolated from KE37 cells by flotation in a sucrose gradient as described in Rios et al. (1992). Prior to further processing, Golgi-enriched fractions were diluted by addition of several volumes of 10 mM Tris-HCl, pH 7.4, and protease inhibitors were added. The suspensions were centrifuged at 200,000 *g* for 2 h at 4°C and pellets were used for subsequent experiments.

Electrophoresis and Immunoblot Analyses

Proteins were separated by SDS-PAGE on 8% acrylamide gels (Laemmli, 1970) and stained with Coomassie blue. Two-dimensional gel electrophoresis of Golgi-enriched fractions was performed according to O'Farrell (1975) and stained with silver nitrate. One- or two-dimensional gels were electrophoretically transferred to nitrocellulose filters according to Towbin et al. (1979). Nitrocellulose filters were blocked for 1 h at 37°C in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk. Then filters were incubated for 1-2 h at 37°C in the primary antibody diluted in TBST, washed in the same buffer, and incubated for 45 min at 37°C with secondary anti-rabbit or anti-human IgG antibodies conjugated with either alkaline phosphatase (Promega Corp., Madison, WI) or peroxidase (Catalg, San Francisco, CA). After washes, alkaline phosphatase activity was revealed with 0.1 M Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ containing both nitroblue tetrazolium and 5-bromo-4-chloroindolyl phosphate. Peroxidase activity was revealed using the ECL system (Amersham Corp.).

Membrane Extraction and Washing Procedures

Golgi vesicles (50 μg protein) were extracted with Triton X-114 as described by Bordier (1981). The detergent phase was taken up directly in SDS sample buffer; the aqueous phase was first concentrated by precipitation with 10% (wt/vol) TCA. Golgi vesicles were also washed with 1 M NaCl, 0.2 M sodium carbonate, pH 11.0, or 6 M urea. After incubation for 30 min on ice, the samples were centrifuged for 1 h at 200,000 *g*. The supernatants (precipitated with 10% TCA) and pellets were processed for SDS-PAGE followed by immunoblotting with either autoimmune serum or anti-GalTf antibody.

Permeabilization Experiments

HeLa cells grown on glass slides at subconfluent densities were washed with PBS and buffer A (25 mM Hepes-KOH, 125 mM potassium acetate, and 2.5 mM magnesium acetate) and then incubated for different times with Streptolysin O (SLO; Burroughs Wellcome, Research Triangle Park, NC) prepared at varying concentrations in buffer A supplemented with 1 mM dithiothreitol. Cold buffer A was added to SLO-treated cells to stop the permeabilization process and cells were washed two times with buffer A containing 1% BSA. Permeabilized HeLa cells were incubated with the autoimmune serum diluted 1/100 in buffer A containing 1% BSA. After that, cells were washed, fixed in methanol at −20°C, and incubated with anti-GalTf. As a control, an analogous experiment was performed in which permeabilized cells were incubated with the anti-GalTf antibody before fixation and thereafter with the autoimmune serum. Permeabilization using 0.5 U/ml SLO for 7 min at 37°C gave the best results and was routinely used.

Electron Microscopy

Staining of myotubes by immunoperoxidase and processing for electron microscopy was carried out according to Saraste et al. (1987). Briefly, cells were fixed in paraformaldehyde-lysine-periodate fixative for 4 h at room temperature, permeabilized with 0.05% saponin, and incubated with anti-p210 IgG (1/100) overnight. Cells were then incubated with peroxidase-conjugated Fab fragments of sheep anti-human IgG for 60 min, followed by reaction with diaminobenzidine and Epon embedding.

Results

The Auto-immune RM Serum Decorates the Golgi Apparatus in a Wide Variety of Cells

The serum from patient R.M., hereafter termed "RM serum", was assayed by indirect immunofluorescence in a variety of mammalian cells (HeLa, KE37, human myoblasts, NRK, and CHO). In all cases, RM serum showed a very high reactivity to the GA. A weaker nuclear staining could also be observed but disappeared at higher dilution of the serum. An example is presented in Fig. 1 where double staining of HeLa cells with RM serum and with a monospecific antibody directed against the *trans*-Golgi marker GalTf is shown (Fig. 1, *A* and *B*). Distributions of both antigens were virtually identical. In human myoblasts decorated with RM serum and with the monoclonal antibody CTR433, a *medial*-

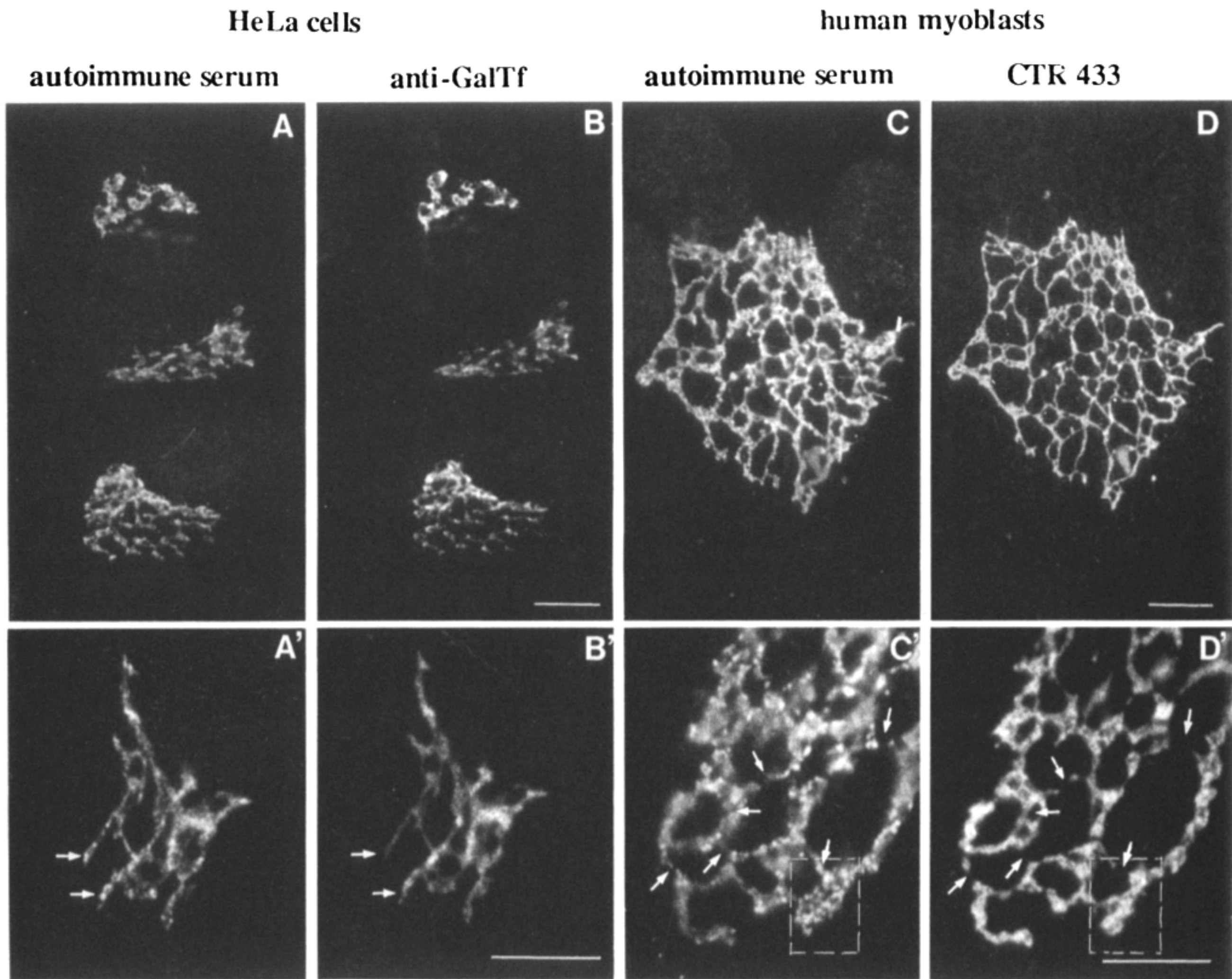

Figure 1. Immunofluorescence microscopy of methanol-fixed HeLa cells (*A* and *B*), human myoblasts (*C* and *D*) stained with RM serum (*A* and *C*), and the Golgi markers anti-GalTf (*B*) and CTR 433 (*D*). In both cell types, RM serum gave a strong fluorescent signal in the Golgi region that colocalized with those shown by the Golgi markers. No staining of the ER or the cytoplasm was detected. A more detailed observation (*A'–D'*) revealed that the staining patterns displayed by RM serum (*A'* and *C'*) and GalTf (*B'*) or CTR 433 (*D'*) were not identical (compare *A'* and *B'* and *C'* and *D'*). The Golgi staining of both Golgi markers was thinner and more uniform than that of RM serum which appeared patchy or vesicular and sometimes larger over the Golgi area. Bar, 5 μm.

Golgi marker (Jasmin et al., 1989), the colocalization of both antigens was also apparently perfect (Fig. 1, *C* and *D*). Despite the low resolution of immunofluorescence microscopy, a close examination of the staining patterns revealed some differences between Golgi markers and RM serum. Notably, the latter showed a rather discontinuous or granular appearance (see Fig. 1, *A'* and *C'*), and was often slightly larger than the staining obtained with Golgi markers. This was even more obvious for myoblasts in which the GA is more extended (Fig. 1, *C'* and *D'*). These observations suggested that RM serum could decorate structures tightly associated to those defined by reference Golgi markers.

Structures Decorated by RM Serum Are Particularly Sensitive to MT-disruption

Segregation between the staining observed with RM serum and that obtained with Golgi markers became evident when cells were treated with nocodazole (Fig. 2). After short treatments of nocodazole (up to 15–30 min), the morphology of the GA visualized with the anti-GalTf antibody was not significantly modified (Fig. 2 *B*). By contrast, numerous small vesicles, or tubules, decorated by RM serum appeared around the GA but also outside the Golgi region (Fig. 2 *A*). By 2–3 h of nocodazole treatment, the GA had completely fragmented and the Golgi elements had dispersed throughout the cytoplasm. In these conditions, staining patterns observed with RM serum and with anti-GalTf were only partially coincident, RM serum recognizing a number of elements that did not contain GalTf (Fig. 2, compare *C* and *D*). Furthermore, a detailed observation revealed that even in elements containing both antigens, the staining patterns were not identical either, RM serum decorating distinct domains often at the periphery of the GalTf-containing domains (see Fig. 2, *E* and *F*, *arrows*).

From this set of experiments, we concluded that RM serum additionally decorates distinct structures closely as-

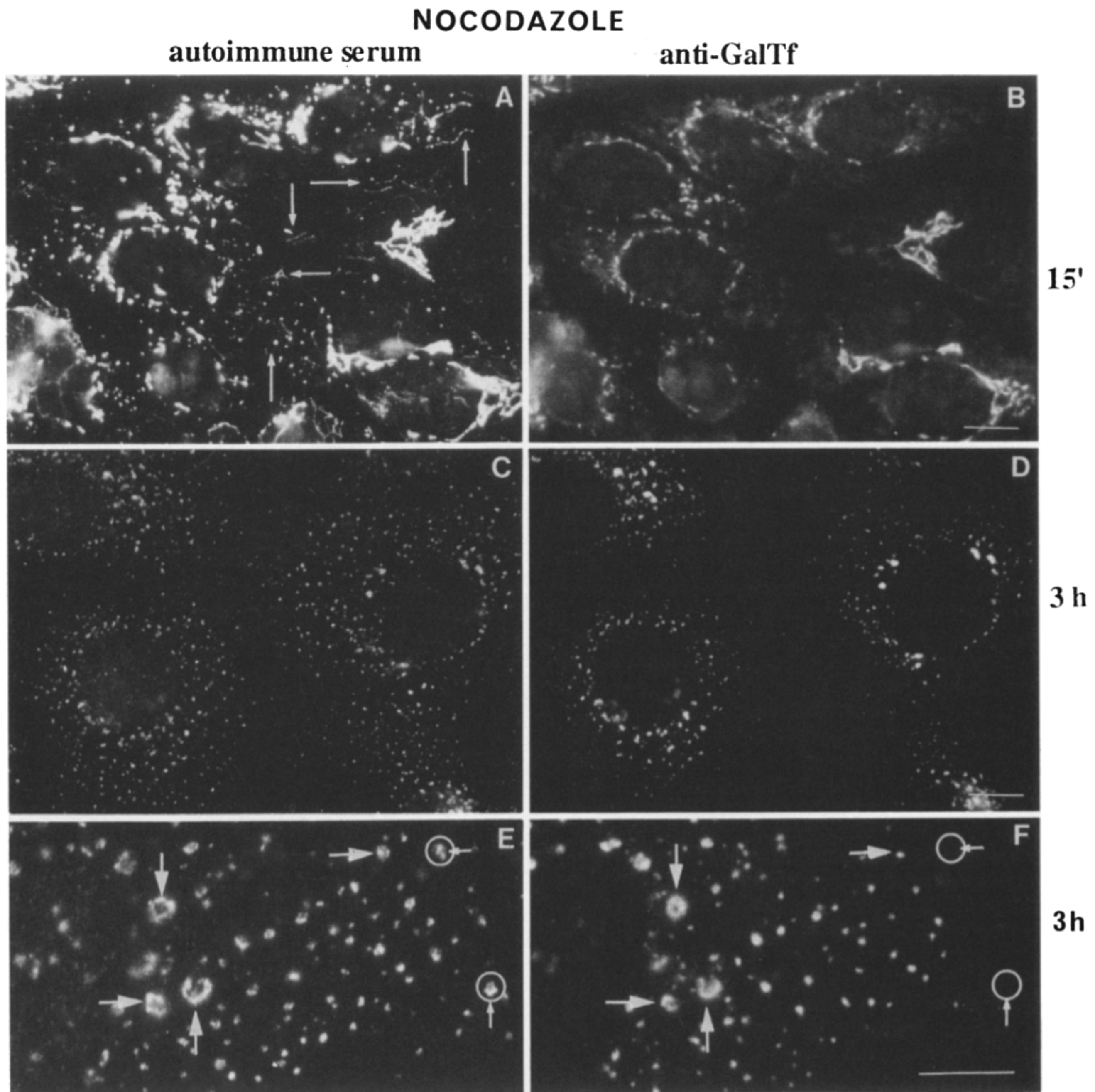

Figure 2. Effect of nocodazole on the distribution of RM serum autoantigen. HeLa cells were treated with 10 μM nocodazole for 15 min (*A* and *B*) and 180 min (*C* and *D*), fixed and double stained with autoimmune RM serum (*A* and *C*) and the anti-GalTf antibody (*B* and *D*). After 15 min of nocodazole treatment numerous vesicles and tubules staining with autoimmune RM serum but negative for GalTf began to distribute throughout the cytoplasm (*A*, *arrows*; this figure has been slightly underexposed to show the small tubules). After 3 h nocodazole, a number of discrete Golgi elements were observed with the anti-GalTf antibody, all of which (*E*, *large arrows*) were also labeled with autoimmune RM serum. Other membranous elements uniquely stained by RM serum were also apparent (*E*, *circles with arrows*). Note that labeling patterns of Golgi elements displayed by both antibodies were not identical, that of RM serum being again more extensive than the pattern produced by the anti-GalTf antibody. Bar, 5 μm.

sociated with the GA and highly dependent on microtubules for both their integrity and their association with the GA.

RM Serum Reacts with a Golgi-enriched 210-kD Protein

In order to characterize the autoantigen recognized by RM serum, the Triton-soluble fraction of metabolically labeled HeLa cells (see Materials and Methods) was used for immunoprecipitation. Immune complexes were analyzed by SDS-PAGE and fluorography. Three major bands of 210, 130, and 45 kD were immunoprecipitated by RM serum (Fig. 3 *A*, lane *1*). Several other bands were also occasionally observed. In control experiments using normal human serum these bands were not detected (Fig. 3 *A*, lane *2*). A complex

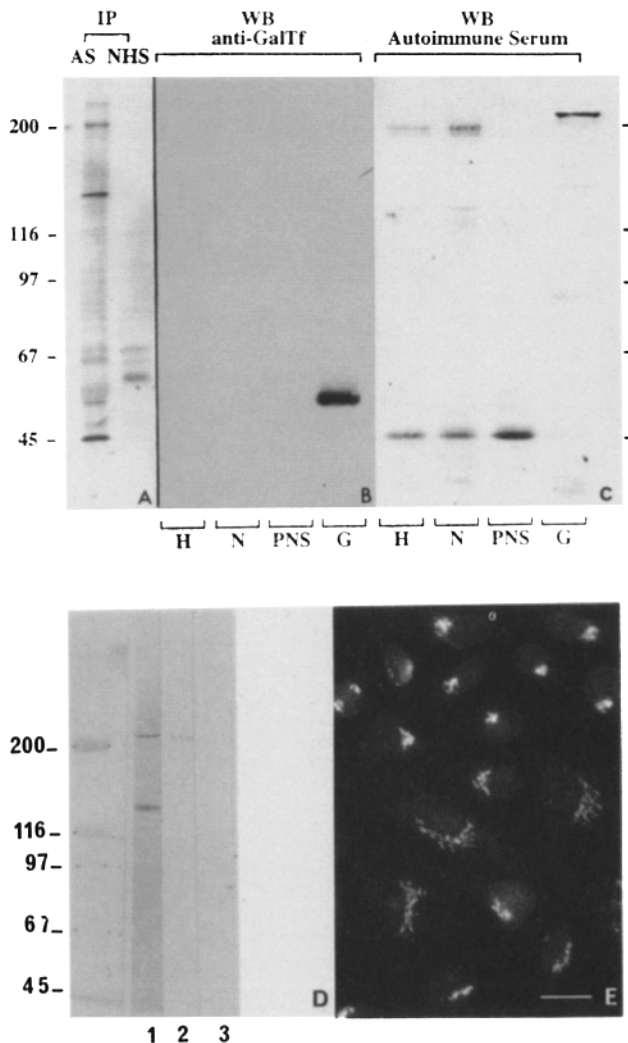

Figure 3. Autoimmune serum recognizes a 210-kD Golgi protein. Immunoprecipitation and Western blotting were used to identify the Golgi antigen of the autoimmune serum. (*A*) HeLa cells were metabolically labeled with ^{35}S-Trans-Label and detergent extracts of cells were used for immunoprecipitation. Autoimmune RM serum (*AS*) immunoprecipitated three major proteins of 210, 130, and 45 kD that did not appear in immunoprecipitates using normal human serum (*NHS*). (*B*) KE37 cells were fractionated and a Golgi-enriched fraction prepared by flotation in a sucrose gradient. The enrichment of different subcellular fractions in Golgi membranes was tested by Western blotting using an anti-GalTf antibody. Proteins from total homogenate (*H*), nuclear pellet (*N*), post-nuclear supernatant (*PNS*), and the Golgi-rich fraction were separated by SDS-PAGE, transferred to nitrocellulose filters, and assayed for their content in GalTf. (*C*) A blot with identical samples to those in (*B*) was incubated with autoimmune RM serum and bound antibody was detected by chemiluminescence. Proteins of 200, 130, and 45 kD were the major proteins observed in homogenate, nuclear pellet, or postnuclear supernatant. In Golgi-enriched fractions only a 210-kD band was recognized by autoimmune RM serum; in some experiments a lighter band with a slightly faster mobility was also detected. (*D*) The pellet obtained by high-speed centrifugation of the postnuclear supernatant, which was highly enriched in proteins of 210 and 130 kD, as judged by Western blotting with RM serum and devoid of any 45-kD signal (lane *1*), was used to affinity-purify RM serum on individual bands. When assayed on Golgi-enriched fraction (lanes *2* and *3*), p210 affinity-purified immunoglobulins reacted only with the 210-kD band (lane *2*) whereas the p130 affinity-purified immunoglobulins did not reveal any band (lane *3*). The MW markers were stained by Ponceau red S. (*E*) p210 affinity-purified immunoglobulins revealed the GA when used for immunofluorescence on HeLa cells after methanol fixation. Bar, 10 μm.

reactive pattern was indeed expected with an autoimmune serum, and in order to ascertain which of these bands corresponded to the Golgi autoantigen, we turned to human lymphoblasts from the KE37 cell line, which provided us, in a reproducible manner, with a Golgi-enriched fraction prepared by flotation on a sucrose gradient (see Rios et al., 1992). Total homogenate, nuclear pellet, postnuclear supernatant (75 μg), and Golgi-enriched fraction (50 μg) were independently examined for their content in Golgi membranes by Western blotting, using an affinity-purified anti-GalTf antibody (Fig. 3 *B*): only the Golgi-enriched fraction contained detectable amounts of GalTf. An identical nitrocellulose filter was incubated with RM serum (Fig. 3 *C*). Reactive bands with apparent molecular masses of 200, 130, and 45 kD were the major components detected in the nuclear pellet or in the postnuclear supernatant of KE37 cells. However, a different pattern was observed in Golgi-enriched fractions: a 210-kD protein, that sometimes appeared as a doublet, was highly reactive (Fig. 3 *C*). These results strongly suggested that the 210-kD band was the Golgi autoantigen. Definitive demonstration of this was obtained by affinity purifying RM serum on individual reactive bands after electrophoretic separation and transfer onto nitrocellulose filters (Fig. 3 *D*). As a source of antigens, we used a membrane-enriched fraction obtained by high-speed centrifugation of the postnuclear supernatant: the 210- and the 130-kD bands were highly enriched in this pellet and were the only reactive bands with RM serum (Fig. 3 *D*, lane *1*), the 45-kD band turning out to be soluble (not shown). Antibodies retro-eluted from each band were further tested on a Golgi-enriched fraction: the 210-kD band was revealed by p210 affinity-purified antibodies (Fig. 3 *D*, lane *2*) whereas the p130 affinity-purified antibodies did not reveal any component in this fraction (Fig. 3 *D*, lane *3*) although it revealed the p130 in the membrane-enriched postnuclear fraction (not shown). Moreover, when assayed by immunofluorescence, p210 affinity-purified antibodies produced a strong Golgi staining pattern on HeLa cells (Fig. 3 *E*), whereas p130 affinity-purified antibodies did not give any Golgi staining nor any obvious other membrane compartment (not shown).

Finally, proteins from the Golgi-enriched fraction were resolved by two-dimensional electrophoresis and were either stained with silver nitrate or transferred to nitrocellulose filters and incubated with the autoimmune serum (Fig. 4). The serum recognized one spot of molecular mass >200 kD and pI 5.6 (Fig. 4 *A*) which corresponded to an abundant protein in silver-stained gels (Fig. 4 *B*).

The 210-kD Protein Is a Peripheral Membrane Protein

The membrane localization of the 210-kD protein was investigated by using different extraction procedures. Isolated Golgi membranes (50 μg) were extracted with 2% Triton X-114 (the most commonly employed method for separating hydrophilic and hydrophobic proteins) or washed with 6 M

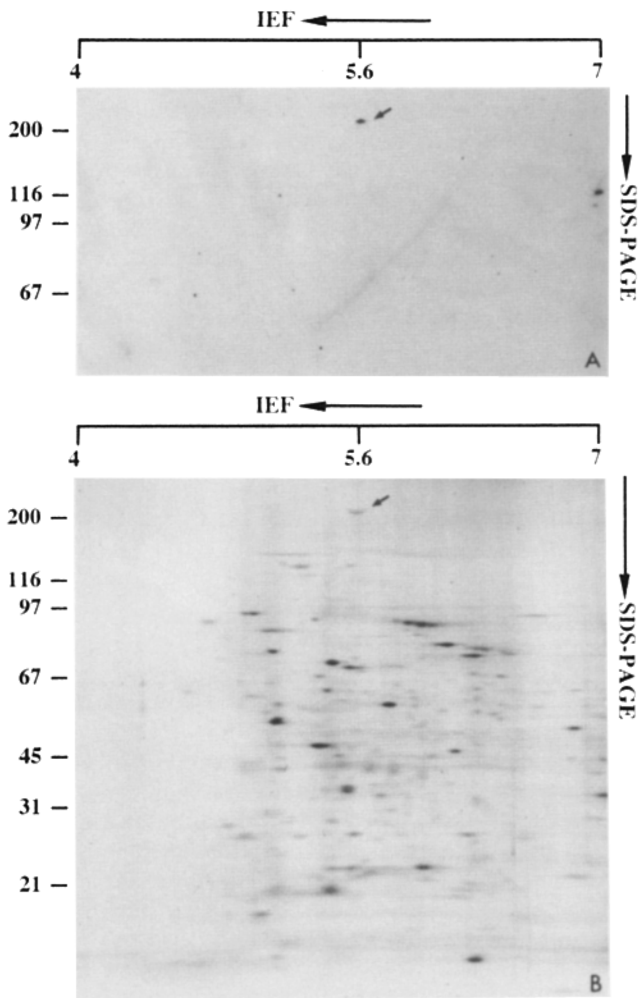

Figure 4. The Golgi-enriched fraction was resolved by two-dimensional electrophoresis. Two-dimensional gels were either transferred to nitrocellulose filters and incubated with autoimmune RM serum (*A*) or stained with silver nitrate (*B*). Autoimmune RM serum recognized a spot of molecular mass 210 kD and pI 5.6.

urea, 1 M NaCl, and 0.2 M sodium carbonate, pH 11.0. Supernatants and pellets of different treatments and aqueous and detergent phases of Triton X-114 extraction were analyzed by SDS-PAGE and transferred to nitrocellulose filters. The upper halves of filters were processed for immunolabeling with RM serum (Fig. 5 *A*). The lower halves of Western blots were immunolabeled with anti-GalTf antibody to monitor the different extraction procedures (Fig. 5 *B*). GalTf is an integral membrane protein disposed at the luminal face of the *trans* cisternae. As expected, GalTf remained associated to membranes after high salt, high pH, and urea treatments and partitioned in the detergent phase of Triton X-114 extraction. In contrast, the 210-kD protein entirely partitioned in the aqueous phase of Triton X-114 extraction and became soluble upon all other treatments, indicating that

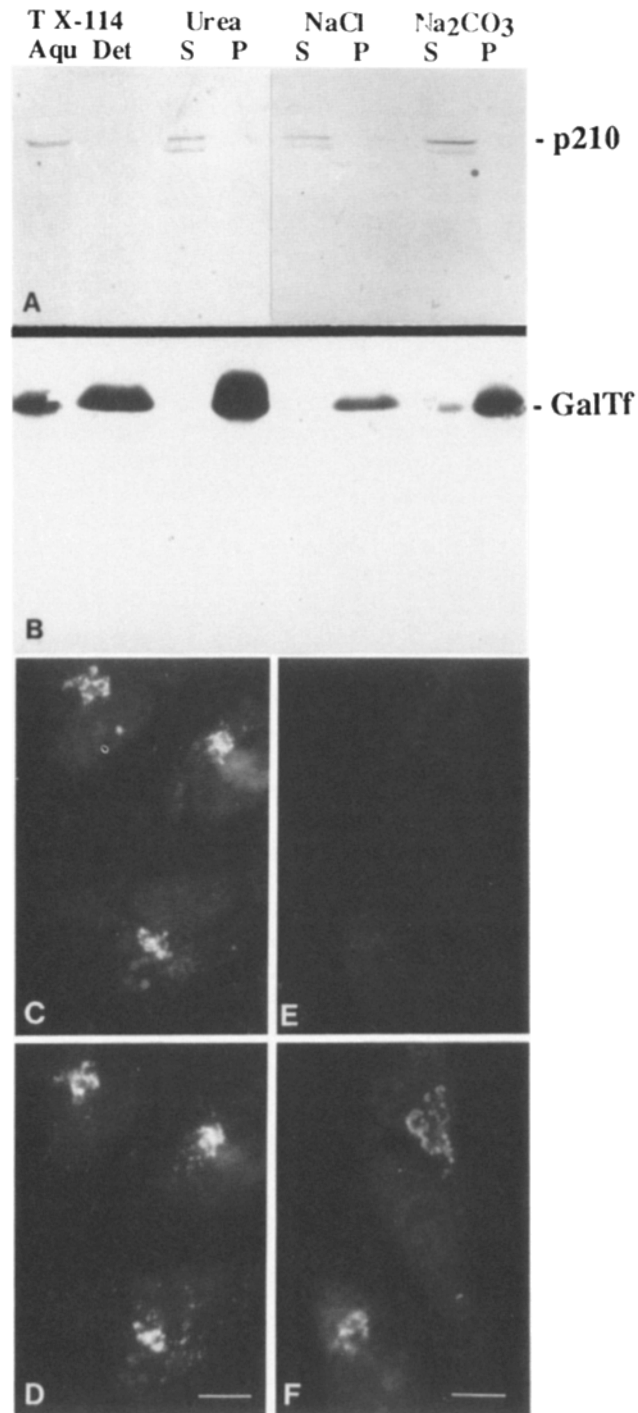

Figure 5. p210 is an external and peripheral protein in Golgi membranes. Golgi membranes were treated with 2% Triton X-114 (*TX-114*) as described by Bordier et al. (1981) and both the aqueous (*Aqu*) and detergent (*Det*) phases were separated by SDS-PAGE and transferred to nitrocellulose filters. Other Golgi membranes were treated with 6 M urea, 1 M NaCl, and 200 mM sodium carbonate (pH 11.0), and after centrifugation, the resulting supernatants (*S*) and pellets (*P*) were processed as described above. The upper half of the filter was assayed by immunoblotting to detect p210 (*A*) and the lower half was incubated with anti-GalTf antibody as a control for a Golgi integral membrane protein (*B*). (*C–F*) HeLa cells were permeabilized using 0.5 U/ml SLO for 7 min at 37°C and then incubated for 1 h with either autoimmune RM serum (*C*) or the anti-GalTf antibody (*E*). After washes, permeabilized cells were fixed and stained for GalTf (*D*) or p210 (*F*), respectively. No staining of the GA was observed in permeabilized cells incubated with anti-GalTf indicating that Golgi membranes remained intact after permeabilization treatment. Bar, 5 μm.

p210 protein is a peripheral cytoplasmically disposed membrane protein.

A different approach was also undertaken to confirm the localization of p210 in Golgi membranes. Semi-intact Hela cells were prepared using the bacterial toxin SLO to selectively permeabilize the plasma membrane to allow the access of antibodies to cytoplasm while maintaining the morphological integrity of the GA. In addition, in cells perforated with SLO, the GA and most intracellular organelles were shown to be well preserved at light and electron microscopic level (Miller and Moore, 1991).

HeLa cells were incubated with 0.2-2 U/ml SLO for 5-10 min at 37°C, washed, and incubated with RM serum for 1 h. Then, cells were washed, fixed, and stained for GalTf. Antibodies bound before and after fixation were visualized using fluorescein and rhodamine secondary antibodies, respectively (Fig. 5, *C* and *D*). Treatment with 0.5 U/ml SLO for 7 min at 37°C gave the best results: more than 80% of the cells were permeabilized and showed Golgi staining after incubation with RM serum for 1 h (Fig. 5 *C*). By contrast, no GA staining was observed in permeabilized cells incubated with anti-GalTf antibody before fixation (Fig. 5 *E*), indicating that the GA itself was not perforated by SLO and remained impermeant to antibody molecules.

p210 Is Concentrated at the Cis Side of the GA

In an attempt to localize p210 at the ultrastructural level, we made use principally of primary cultures obtained from human muscle biopsies that are capable of producing in vitro myogenesis. Although this system was not tractable for immunoelectron cryomicroscopy, therefore imposing localization by immuno-peroxidase technique, it had two significant advantages: (*a*) it provided us with mononucleated cells displaying a highly extended GA; it was in these cells that RM serum decorated the GA with the most granular aspect (see Fig. 1, *C* and *C'*), suggesting that one could hopefully identify the reactive structures within the GA; and (*b*) the GA in differentiating myotubes displays a characteristic perinuclear distribution, in which the *cis-trans* polarity is easily identifiable from the nuclear periphery towards the plasma membrane (Tassin et al., 1985*b*).

In mononucleated cells p210 demonstrated a clear asymmetrical distribution on one side of the GA (Fig. 6), the labeling being externally associated with budding or tubular processes in zones which, from their aspect, could correspond to the so-called "non-compact zones" described by Rambourg and Clermont (1990) (Fig. 6, *A* and *B*, *curved arrows*). Another constant feature was that face views of Golgi membranes demonstrated a discontinuous appearance of the labeling, corresponding to more or less regularly spaced small cavities (Fig. 6 *A*, *straight arrows*), which could correspond to the granular aspect observed at the optical level. Higher magnification suggested that these cavities corresponded to large perforations of the saccules of the mid compartment described as "wells" by Rambourg and Clermont (1990) in the so-called "compact zones" of the GA. A face and a side view from the same Golgi area are indicated in Fig. 6 *B* (*straight arrows*).

By immunofluorescence microscopy, young myotubes were decorated by RM serum in a manner strikingly similar to that obtained with any Golgi marker, i.e., essentially as a perinuclear ring (Fig. 7 *A*; for comparison see Tassin et al., 1985*b*). One could, however, observe a rather punctuated aspect of the perinuclear GA when decorated with RM serum (Fig. 7 *A*, *arrows*). Ultrastructural localization demonstrated that p210 was associated with tubulovesicular structures at the *cis* side of the GA, and next to the nuclear envelope, which could penetrate deeply between saccules, the precipitate being detected between the lateral sides of the Golgi stacks. In these perforations, the labeling appeared abundant at the edge of the cisternae (Fig. 7 *C*, *arrows*).

Overall, despite the difficulty of precisely deciding the extent of the peroxidase labeling, the observation of numerous sections led us to conclude that p210 could interact with the external side of an abundant tubulovesicular system on the *cis* side of the Golgi, and that this system could encompass, or extend to, connecting structures or vesicles between individual saccules and dictyosomes.

p210 Redistributes in the Intermediate Compartment upon BFA Treatment

BFA treatment of cells results in the rapid loss of the Golgi as a distinct organelle due to the redistribution of Golgi content and membranes into the ER. Double immunofluorescence staining with RM serum and with the *trans*-Golgi marker GalTf was performed on BFA-treated HeLa cells to determine the fate of p210. Within 5 min of BFA treatment (1 μg/ml), tubular processes extending out of enlarged Golgi structures were visible with the anti-GalTf antibody (Fig. 8 *B*). RM serum showed a similar pattern although the tubular extensions were less evident (Fig. 8 *A*). After 30 min of BFA treatment, in most cells GalTf was redistributed in a fine reticular pattern characteristic of ER labeling (Fig. 8 *D*). By contrast, p210 was present in numerous small vesicles scattered throughout the cytoplasm (Fig. 8 *C*), a pattern that remained unchanged upon longer incubations with BFA. Under these conditions the staining patterns displayed by GalTf and p210 were completely distinct whereas they were congruent in control cells.

The effect of BFA on p210 was entirely reversible. HeLa cells treated with BFA for 1 h were allowed to recover during 10 or 30 min in BFA-free medium. 10 min after removal of the drug, p210-containing vesicles appeared larger and tubular structures extending towards a perinuclear region could be observed (Fig. 8 *E*). After 30 min of recovery, the spotty staining was greatly reduced and p210 was concentrated in a compact structure close to the nucleus (Fig. 8 *G*). The relocalization of GalTf was significantly slower although some vesicles could be observed 10 min after removal of the drug (Fig. 8 *F*). These vesicles always colocalized with p210-containing elements, suggesting that movement of proteins out of the ER to the GA occurred through these elements. After 30 min of BFA recovery, both proteins colocalized again (Fig. 8 *H*). The time course of the relocalization of both proteins strongly suggested that p210 elements were involved in the repositioning of the Golgi complex after removal of BFA, a possibility which would fit with the localization of p210 in the CGN.

Until now, three proteins have been described to exhibit a similar staining pattern after BFA treatment: p58 (Saraste and Svensson, 1991), p53 (Lippincott-Schwartz et al., 1990), and p23 (Luen Tang et al., 1993), all of which reside in the intermediate compartment. To determine the nature of the spotty staining displayed by p210, we compared the localiza-

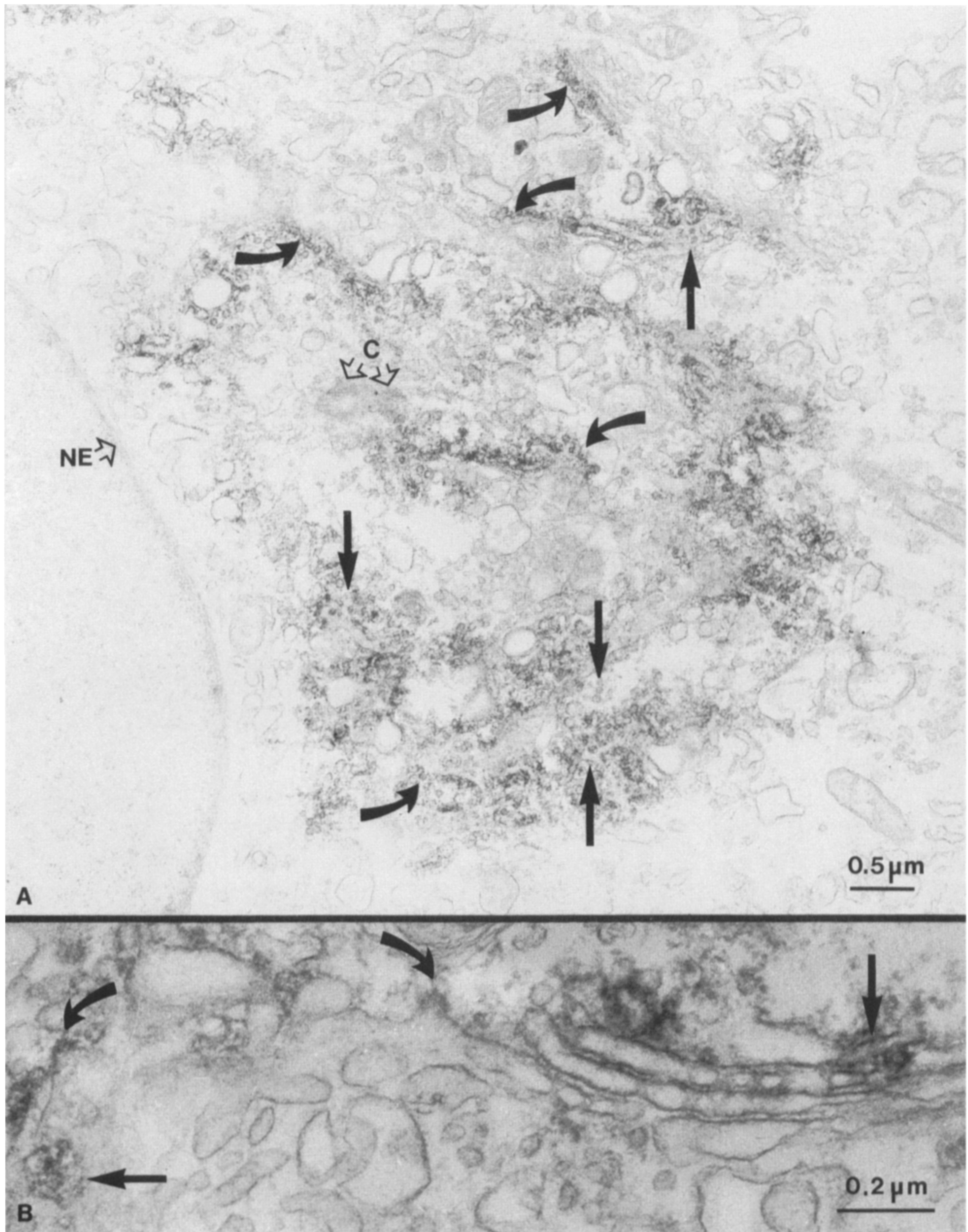

Figure 6. Ultrastructural localization of p210 in mononucleated cells from human myogenic cultures, using indirect immunoperoxidase techniques. At low magnification (*A*), the GA displays a large spatial extension on one side of the nucleus (the nuclear envelope is indicated in *A*, *NE*) and surrounds the centrosome (the two centrioles are indicated in *A*, *C*). Side views of the Golgi elements demonstrate that p210 is associated with one side of the GA, where particularly tubular membrane extensions, or vesicles, appear labeled (*curved arrows*). Face views demonstrate numerous and rather even-spaced spots of regular size (*straight arrows*) which look like cavities. On higher magnification (*B*), and although ultrathin sections do not allow one to easily appreciate the overall architecture of the GA, one can recognize such cavities on face view (*horizontal straight arrow*) and tentatively identify them on side views (*vertical straight arrow*). They apparently correspond to perforations in register, or wells, which often interrupt the mid saccules (Rambourg and Clermont, 1990) and their decoration by RM serum could be responsible for the granular aspect of immunofluorescent staining at the light level (see Fig. 1). Curved arrows in *B* point to labeled budding elements.

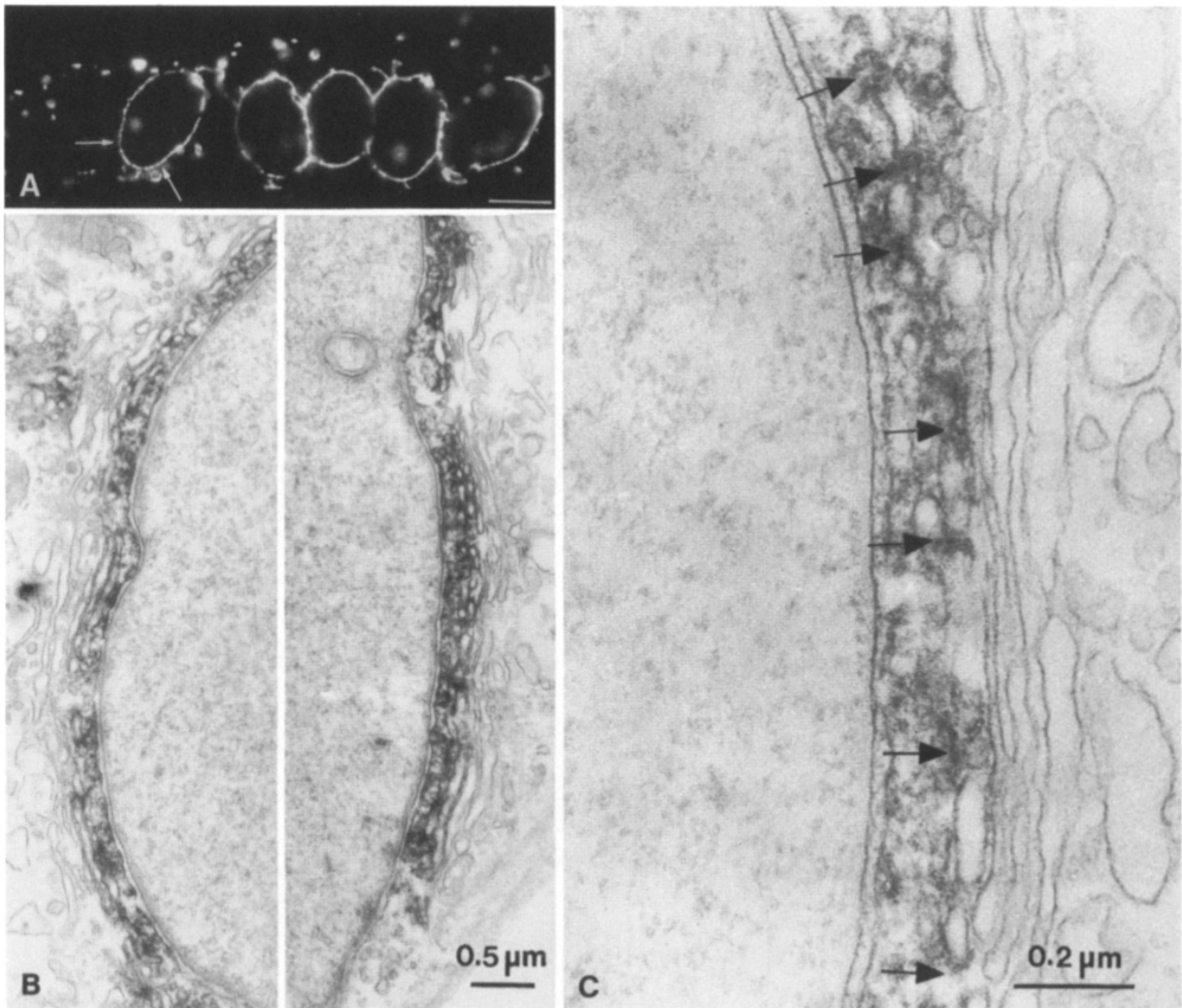

Figure 7. Immunolocalization of p210 in myotubes from human myogenic cultures. (*A*) Immunofluorescent staining. RM serum decorates the periphery of the syncytium nuclei, and some elements within the cytoplasm, in a manner strikingly similar to what is observed with bona fide Golgi markers (see Tassin et al., 1985*b*). Note however that the perinuclear staining, when in focus, appears patchy (*arrows*), a feature which was not observed with other markers of the Golgi. (*B* and *C*) Two magnifications of the ultrastructural localization of p210 in the perinuclear GA, using indirect immunoperoxidase techniques. They demonstrate unambiguously that p210 is on the nuclear side of the GA, which has been previously demonstrated to be the *cis* side (Tassin et al., 1985*b*). The labeling apparently concerns an abundant tubulo-vesicular system on the *cis* side of the Golgi saccules proper, but can also penetrate into zones where saccules are interrupted (*C*, *arrows*). The last feature is probably responsible for the patchy aspect of the perinuclear staining observed by immunofluorescence (*A*, *arrows*). The low magnification in *B* corresponds to the same nucleus for which most of the chromatin has been deleted on the picture for the sake of space.

tion of p58 and p210 in control conditions and after BFA treatment. As shown in the double label immunofluorescence experiment in Fig. 9, p58 showed a larger distribution than p210 in both myoblasts (Fig. 9, *A* and *B*) and HeLa cells (Fig. 9, *C* and *D*): codistribution of p58 and p210 was restricted to the Golgi structure (Fig. 9, *A* and *B*, *arrows*), but vesicular elements decorated by p58 were observed outside the Golgi region in both cell types. By contrast, upon incubation of cells with BFA for 1 h, both p210 and p58 were apparently present in the same structures (Fig. 9, *E* and *F*). Incubations of cells for 90 min at 16° did not modify the Golgi staining pattern exhibited by p210 although tubules emerging from the Golgi region are sometimes visible with the RM serum (Rios, R. M., and M. Bornens, manuscript in preparation).

From these observations, we could conclude that, rather than being a resident protein of the intermediate compartment such as p58 (Saraste and Svensson, 1991), p210 is a Golgi protein which is induced to redistribute in the intermediate compartment by BFA. To clearly demonstrate that BFA-induced p210-containing vesicles corresponded to the intermediate compartment, we took advantage of the findings of Lippincott-Schwartz et al. (1990) who reported that the intermediate compartment remains intact in BFA-treated cells, the Golgi resident proteins cycling continuously, in a

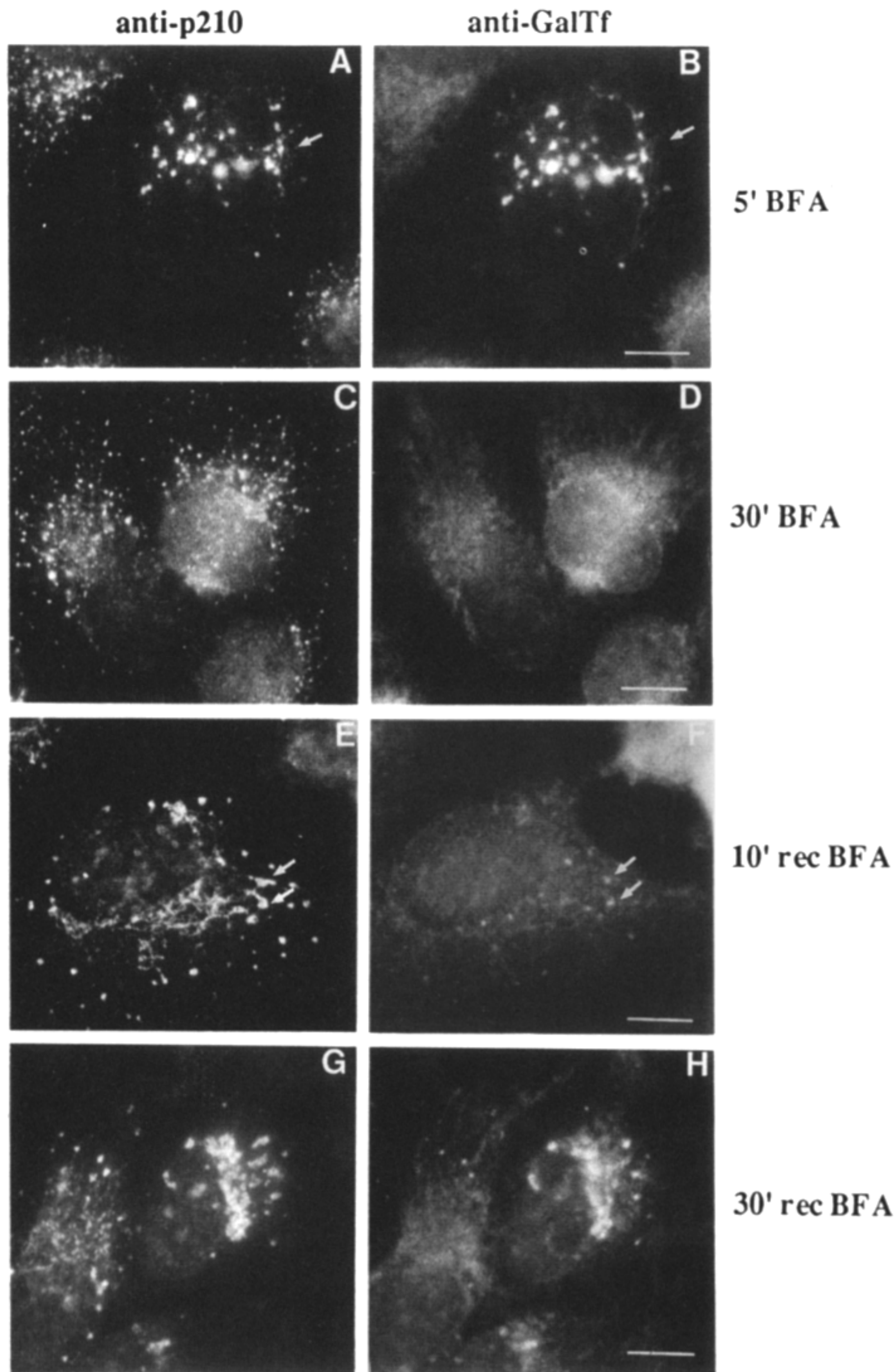

Figure 8. Immunofluorescence localization of p210 in BFA-treated cells. (*A–D*) HeLa cells were incubated with BFA (1 μg/ml) for 5 (*A* and *B*) or 30 min (*C* and *D*) at 37°C before fixation and dual immunofluorescence staining with autoimmune RM serum (*A* and *C*) or anti-GalTf (*B* and *D*). Treatment of cells with BFA for 5 min caused the cisternae to enlarge and to extend thin tubular processes out from the Golgi region. At this time, the p210 protein still overlapped with GalTf. After 30 min of treatment, p210 and GalTf were no longer codistributed. GalTf showed a reticular staining characteristic of ER redistribution whereas p210 appeared in numerous vesicles scattered throughout the cytoplasm. (*E–H*) Alternatively, HeLa cells were incubated for 2 h with BFA and then chased in fresh, untreated medium for 10 (*E* and *F*) or 30 min (*G* and *H*) to allow recovery from BFA treatment. The cells were then fixed and labeled for p210 and GalTf. Note the faster reorganization of the vesicular elements containing p210 as compared with GalTf and the extension of tubular processes from these elements. Bar, 5 μm.

microtubule-dependent manner, between ER and this compartment. HeLa cells incubated with 1 μg/ml BFA for 1 h (Fig. 10, *A* and *B*) and further with BFA plus 10 μM nocodazole for 2 h (Fig. 10, *C* and *D*) were double stained for p210 and GalTf. Whereas GalTf was localized in a reticular pattern in BFA-treated cells (Fig. 10 *B*), it appeared in some of the vesicular structures containing p210 after incubation with BFA plus nocodazole (compare Fig. 10, *B* and *D*). These vesicles were significantly larger than those observed in cells treated with BFA alone (compare (Fig. 10, *A* and *C*) probably due to the accumulation of Golgi proteins, such as GalTf, moving out of the ER and incapable of returning to the ER.

This effect was indeed dramatically enhanced when BFA-treated cells were incubated with the calcium ionophore A23187 (Fig. 11), an agent that has been reported to stimulate the secretion of ER-resident proteins by perturbing the normal sorting system (Booth and Koch, 1989). Addition of ionophore A23187 to cells modified the morphology of neither the Golgi apparatus (Fig. 11, *A* and *B*) nor the ER visualized using an ER-specific antibody (Fig. 11 *C*). The microtubule network also remained intact under these condi-

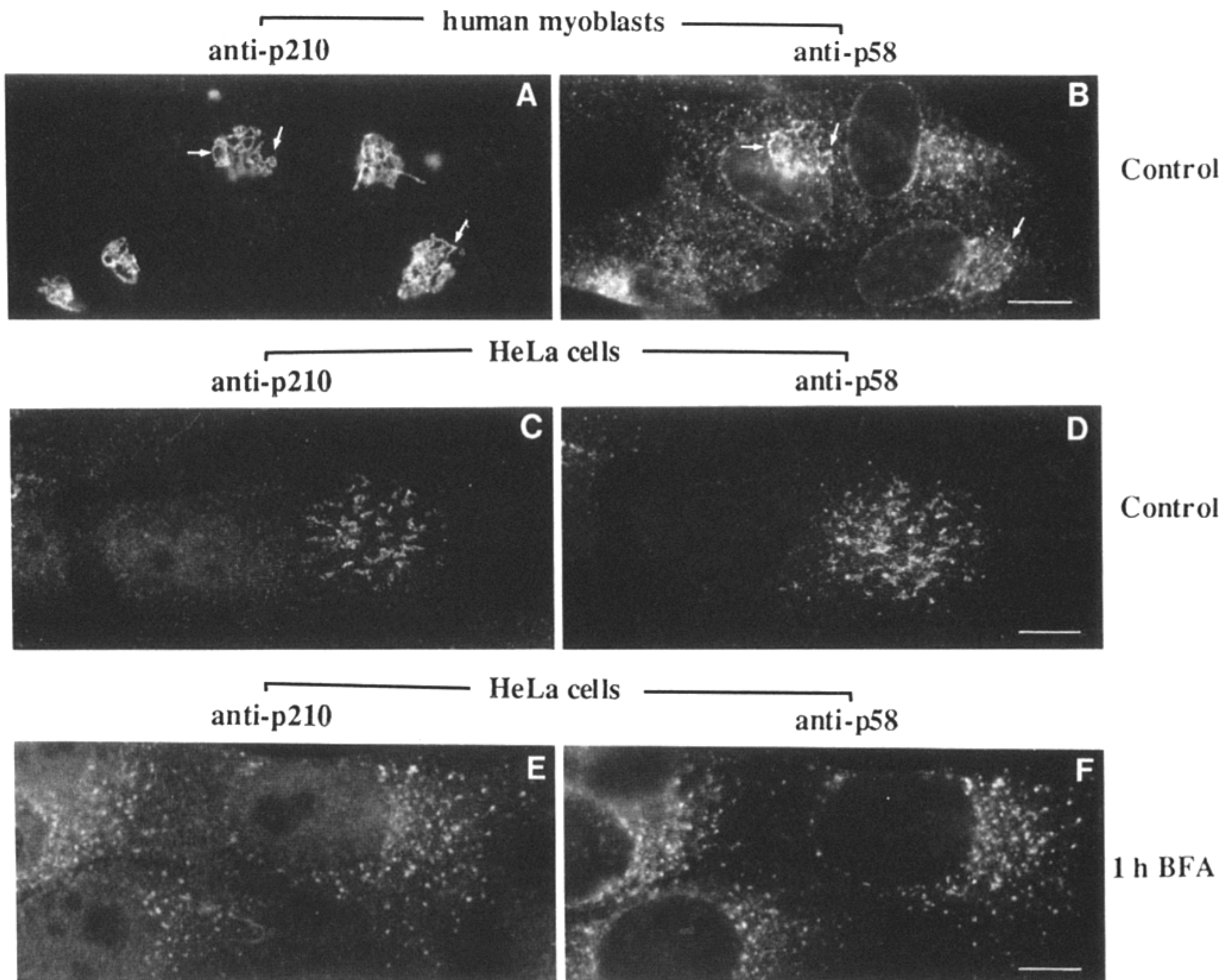

Figure 9. Double immunofluorescence staining of human myoblasts (*A* and *B*) and HeLa cells (*C* and *D*) with autoimmune RM serum (*A* and *C*) and p58, a marker of the intermediate compartment (*B* and *D*). In both cell types, p58-stained Golgi complex (*A* and *B*, *arrows*) and a number of vesicles dispersed throughout the cytoplasm whereas p210 only decorated the GA. After incubation of cells with BFA for 1 h (*E* and *F*), both proteins seem to colocalize in the same vesicular structures. Bar, 5 μm.

tions (not shown). Treatment of cells with BFA followed by BFA plus ionophore A23187 caused the same effect as treatment with BFA plus nocodazole: GalTf accumulated in the vesicles labeled with p210 (Fig. 11, *D* and *E*). In these conditions, the ER appeared fragmented (Fig. 11 *F*). If, in addition, nocodazole was added, practically all GalTf was absent from the ER and appeared in large structures that contained p210 (Fig. 11, *G* and *H*). The effect of nocodazole was not apparently modified by ionophore A23187 and all microtubules became depolymerized (Fig. 11, *I* and *L*). Remarkably, the staining patterns of GalTf and p210 in these conditions were completely coincident. Moreover, ER-resident proteins also appeared to be induced to exit the ER in these conditions as they accumulated in p210-containing vesicles (Fig. 11, *J* and *K*). These observations support and extend those reported by Lippincott-Schwartz et al. (1990) concerning the movement of proteins out of ER in the presence of BFA.

Discussion

Many studies have now shown that human autoantibodies recognize highly conserved antigenic determinants that usually correspond to functionally important domains on molecules. RM serum, a Sjögren's syndrome serum, showed a very high reactivity to the Golgi complex in a wide variety of cells. Circulating autoantibodies reacting against the Golgi complex have been previously described (Rodriguez et al., 1982; Fritzler et al., 1984; Blaschek et al., 1988; Gaspar et al., 1988) but autoantigens were not further investigated. Only one Golgi autoantigen, a protein of molecular mass 230 kD has been recently identified and associated with the *trans* face of the GA (Kooy et al., 1992).

RM serum allowed us to identify a novel Golgi protein designated as p210, which might be highly conserved, as RM serum decorates the GA in many species, including primates, rodents, and amphibians. The high reactivity and specificity exhibited by RM serum in immunofluorescence experiments led us first to attempt to identify the Golgi autoantigen by immunoprecipitation of total cellular extracts. We found, however, that RM serum immunoprecipitated a range of cellular antigens from 45 to 230 kD indicating that multiple autoreactivities were present in the serum. This

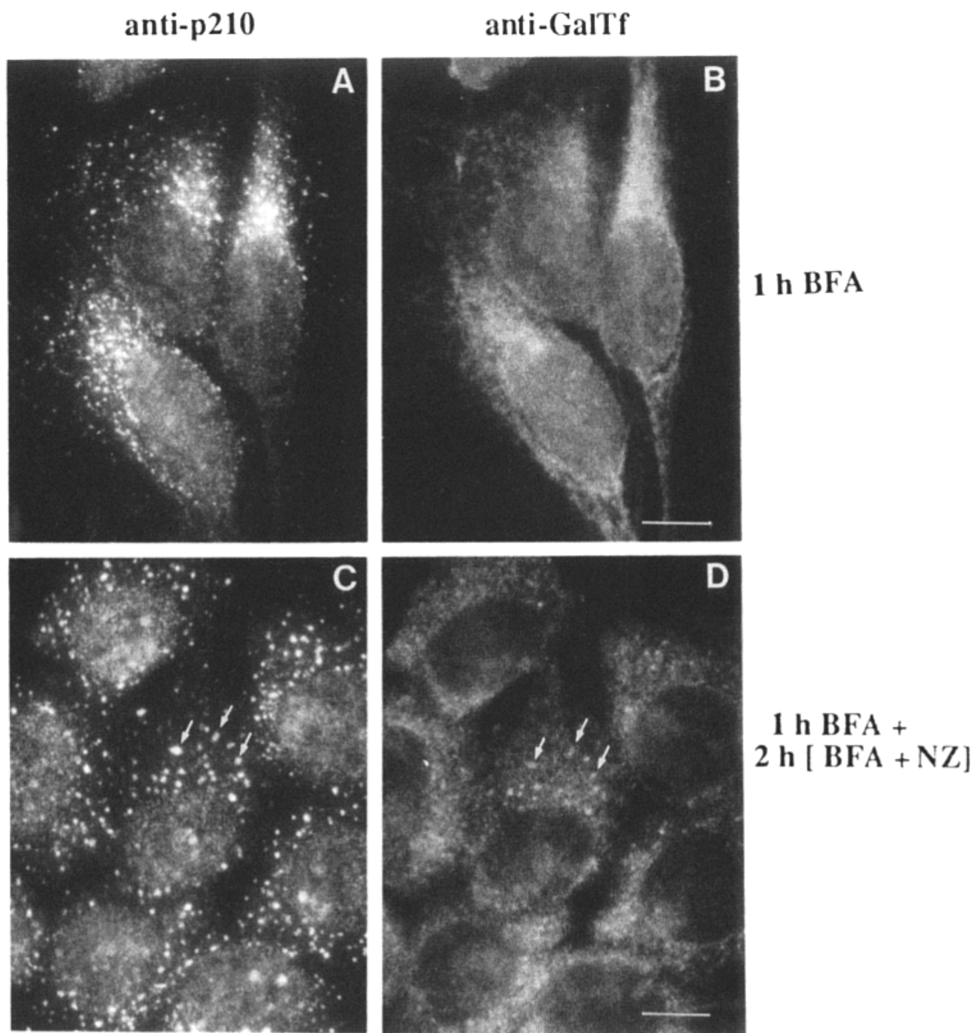

Figure 10. Effect of nocodazole on the distribution of p210 and GalTf in BFA-treated cells. HeLa cells were treated with BFA for 1 h (*A* and *B*) or with BFA for 1 h and then BFA plus nocodazole for 2 h (*C* and *D*). These cells were then fixed and stained for immunofluorescence microscopy using antibodies against p210 (*A* and *C*) and GalTf (*B* and *D*). Upon addition of nocodazole to BFA-treated cells, the staining pattern of GalTf redistributed from a fine reticular pattern characteristic of ER labeling into larger vesicles that contained p210 (*C* and *D*, *arrows*). Bar, 5 μm.

diversity of autoantibody species is a common characteristic of autoimmune diseases (Tan, 1991). The Golgi autoantigen recognized by RM serum was identified as a component with an apparent molecular mass of 210 kD based on two observations: (*a*) the 210-kD band was highly enriched and was the only reactive one detected in a subcellular fraction rich in Golgi membranes; and (*b*) only the antibodies affinity purified on this band decorated the Golgi apparatus by immunofluorescence. The other bands detected by Western blot or by immunoprecipitation must be recognized by distinct autoantibodies present in the serum.

The p210 protein was shown to be a peripheral cytoplasmically disposed membrane protein by two criteria. It partitioned into the aqueous phase on extraction with Triton-X114 and was removed from the membranes by washing with urea, sodium carbonate, and NaCl. Furthermore, p210 was accessible to autoantibodies from the cytoplasm in conditions in which integrity of Golgi membranes was retained. Immunoperoxidase labeling at electron microscopic level using differentiating human myoblast culture confirmed the peripheral cytoplasmically disposed membrane location of the p210. The fenestrated appearance of membranes labeled with p210 in both myoblasts and myotubes, analogous to a tubular network, suggests that these structures correspond to the CGN. That p210 decorated tubulovesicular structures close to the nuclear envelope in myotubes unequivocally established that p210 associates with the *cis* side of the Golgi complex. Interestingly, p210 labeling in myotubes was observed at the rims of some cisternae more distal to the nuclear envelope (probably the *cis* cisternae of the *medial*-Golgi complex) and even at membrane regions where buds seem to emerge. Face views of the Golgi membranes in myoblasts which demonstrated accumulation of labeling into rather regularly spaced wells (see Fig. 7) were consistent with this possibility. They also give an interpretation for the granular immunofluorescence pattern of p210 observed in myotubes, myoblasts, HeLa cells, and many other cell types (not shown): such a granular appearance reflects discontinuous accumulation of the antigen. Taken together, the data are consistent with the possibility that p210 could interact with the external side of an abundant tubulovesicular system on the *cis* side of the Golgi complex, corresponding to the CGN. This network appeared to encompass, or extend to, a subset of vesicular elements within the wells which interrupt the saccules of the mid compartment (in the so-called compact zones) and connecting structures between individual dictyosomes (in the so-called non-compact zones). This conclusion is also supported by the intriguing behavior of p210 after microtubule disruption, namely its partial segregation from Golgi markers.

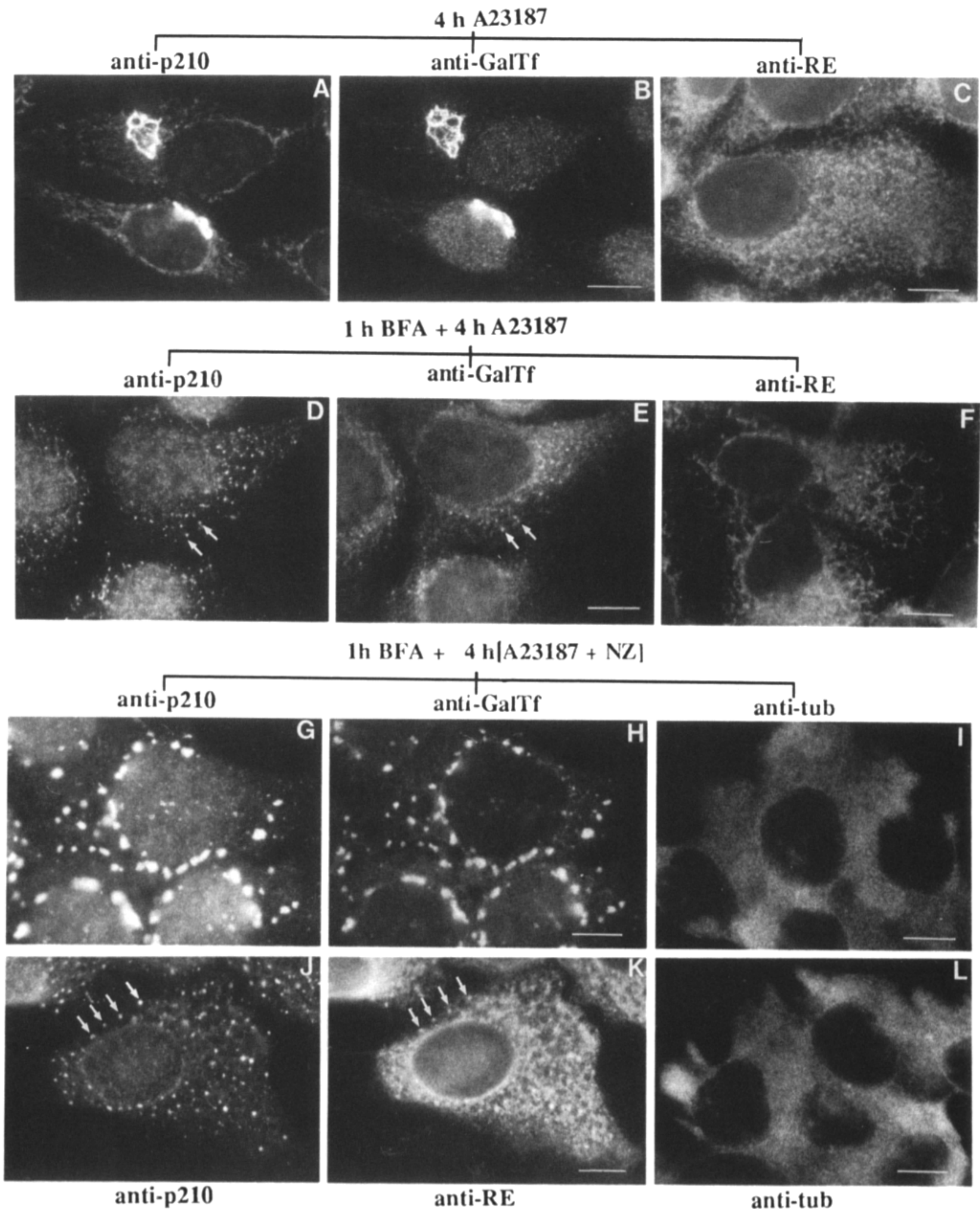

Figure 11. Effect of the calcium ionophore A23187 and nocodazole on the relocalization of both Golgi and ER proteins from ER to p210-containing elements in BFA-treated cells. (*A–C*) HeLa cells were incubated for 4 h with 5 μM A23187 and then fixed and stained for p210 (*A*), GalTf (*B*), or an ER-resident protein (*C*). No changes in the morphology of the Golgi apparatus or the ER were detected. (*D–L*) Alternatively, cells were incubated with BFA for 1 h and then for 4 h with either the calcium ionophore A23187 (*D–F*) or the calcium ionophore plus nocodazole (*G–L*) in BFA-containing medium. Cells treated with BFA and then with BFA plus ionophore A23187 were double stained with autoimmune RM serum (*D*) and anti-GalTf antibody (*E*). As can be seen, ionophore A23187 induced the exit of GalTf from the ER and its accumulation in vesicles labeled with p210 (*arrows*). Morphology of the ER in these conditions was also examined (*F*). In similar experiments, cells were treated with BFA and then with ionophore A23187 plus nocodazole in the presence of BFA in order to induce the exit of proteins from the ER and to block their returning to the ER. Cells were double stained for p210 (*G*) and GalTf (*H*) or for p210 (*J*) and an ER-resident protein (*K*). As expected, ionophore A23187 did not affect the microtubule depolymerization caused by nocodazole (*I–L*). Note that in these conditions, part of the ER proteins themselves accumulate in p210-containing vesicles (*J–K*, *arrows*). Bar, 5 μm.

In many cellular systems, nocodazole induces the breaking of the Golgi complex into hundred of fragments distributed throughout the cytoplasm, and electron microscopic examination has revealed that the fragments are composed of stacked cisternae that maintain their original organization (dictyosomes) (Robbins and Gonatas, 1964; Pavelka and Ellinger, 1983; Thyberg and Moskalewski, 1985; Turner and Tartakoff, 1989). Despite the fragmentation of the GA cellular secretory and biosynthetic activities are remarkably unaffected (Rogalski and Singer, 1984; Rogalski et al., 1984). In immunofluorescence experiments, proteins distributed in different cisternae of the Golgi complex colocalize in the same fragments after disruption of microtubules by drugs (Yuang et al., 1987; Turner and Tartakoff, 1989). On the other hand, proteins, such as p58 or p53, residing in the intermediate compartment, show, in untreated cells, a distribution distinct from that of Golgi markers. It was therefore surprising that p210 segregated from *medial* and *trans* markers in nocodazole-treated cells, whereas the staining patterns were coincident in control conditions. Moreover, this segregation started quite early after nocodazole addition (5–15 min), indicating that p210-specific elements were specially sensitive to microtubule disruption.

The Golgi complex is normally composed of stacks of cisternae interconnected by tubular structures that have been named intersaccular connections (Rambourg and Clermont, 1990). Using NBD–ceramide, Cooper et al. (1990) showed that Golgi cisternae extend tubulovesicular processes along microtubules which form stable contacts with the membranes of adjacent Golgi elements producing the fusion of the cisternae into a continuous reticulum. It has been proposed that these tubular connections are severed after microtubule depolymerization leading to dispersal of individual stacks (Tassin et al., 1985*b*). Turner and Tartakoff (1989) analyzed the dispersal of the GA and distinguished three steps: (*a*) microtubule depolymerization, (*b*) Golgi fragmentation (30–60 min), and (*c*) fragment dispersal (120 min). Disruption of microtubules could produce the vesicularization of the tubular microtubule-dependent intersaccular connections before the fragmentation process became apparent. In our opinion, the results on p210 distribution in nocodazole-treated cells suggest that this protein might be localized both in dictyosomes, in which it associates with the CGN, and in tubular connections which are stabilized by microtubules, a possibility which agrees with the heterogeneous staining displayed by RM serum. If this is true, most of the Golgi proteins would be excluded from these interconnecting tubules since proteins exhibiting this behavior have not been described.

Alternatively, since p210 seems to be associated with the whole CGN, it is possible that among the vesicles observed after short treatments with nocodazole, many arise by fragmentation of tubules emanating from this network. If so, it would mean that the CGN itself is also stabilized by microtubules.

Also surprising was the finding that p210 did not redistribute in the ER in the presence of BFA. On the contrary, it appeared in vesicular elements dispersed throughout the cytoplasm. This result led us to consider whether p210 could be a resident protein of the intermediate compartment like p58 or p53. Double labeling using p58 as a marker confirmed that p210 is primarily bound to the Golgi complex itself rather than to the intermediate compartment. After treatment of cells with BFA, however, p58 and p210 colocalized. We confirmed the identity of the vesicular elements containing p58 and p210 by using drugs that have been reported to block the retrograde transport from the intermediate compartment to the ER such as nocodazole, to stimulate the exit of proteins from ER such as calcium ionophores, or both. Our results are in agreement with those reported previously indicating that (*a*) a cycling pathway between the ER and the intermediate compartment occurs in the presence of BFA (Lippincott-Schwartz et al., 1990); (*b*) the retrograde side of the cycling pathway is inhibited by microtubule disruption (Lippincott-Schwartz et al., 1990); and (*c*) calcium ionophores induce proteins to escape from the ER (Booth and Koch, 1989). Combination of both treatments in the presence of BFA results in the depletion of Golgi proteins from the ER and their accumulation in the intermediate compartment. Resident proteins of ER themselves also accumulated in these structures. These results indicate that p210 is a *cis*-Golgi protein (probably a CGN marker) that redistributes in the intermediate compartment by effect of BFA. Some Golgi proteins, p210 at least, can be excluded from the BFA-induced pathway that carries the Golgi membranes to the ER and suggest the existence of mechanisms that retain specifically certain proteins in the intermediate compartment.

Studying the p210 behavior, it appears clear however that p210-associated structures are involved in the reassembly of the GA following its disruption by BFA. Within minutes of removing the drug, the Golgi markers move out of the ER into peripheral intermediate compartment sites (Lippincott-Schwartz et al., 1990; Alcalde et al., 1992). If microtubules are disrupted during removal of BFA, Golgi structures that can secrete proteins remain localized in these peripheral intermediate compartment sites (Lippincott-Schwartz, 1993; Alcalde et al., 1992). Biogenesis of the Golgi complex involves, therefore, both the intermediate compartment and microtubules. Our results on the recovery of BFA-treated cells are consistent with this scheme. Shortly after removing BFA, tubules containing p210 extended from these intermediate compartment elements and seemed to converge to a perinuclear position. At that moment, *medial* markers (not shown) and *trans*-Golgi markers (Fig. 8, *E* and *F*) began to accumulate into some peripheral p210-containing vesicles.

Formation at 16°C of membrane tubules that contained p53 has been previously reported but resident Golgi proteins appeared to be excluded from these tubular processes (Lippincott-Schwartz et al., 1990; Hauri and Schweizer, 1992). Addition of BFA resulted in the movement of Golgi proteins into the p53-containing tubules. These observations have led to the view that these tubular processes function under both normal conditions and BFA treatment and that BFA could interfere with the mechanism that restricts entry of membrane proteins into the retrograde tubules. Available data support a role of tubular membrane structures in the retrograde transport under both normal conditions and BFA, in the reorganization and repositioning of the Golgi complex after removal of BFA and in connecting homologous cisternae of different stacks or even different cisternae of the same stack (Klausner et al., 1992; Mellman and Simons, 1992; Lippincott-Schwartz, 1993). We have detected the presence of p210, a resident Golgi protein, in membrane tubules which form at 16°C (Rios, R. M., and M. Bornens, manuscript in

preparation) and in those which mediate the reconstruction of the GA after BFA treatment (this work). In addition, p210 appears to be associated with the CGN, a tubular-cisternal network, and presumably with tubules connecting different Golgi stacks. From these results there emerges a relation of this protein with the formation of microtubule-dependent membrane tubules or with processes mediated by them. Since human autoantibodies are usually capable of inhibiting the functional activity of the antigens, the permeabilization experiments used in this work (Fig. 5) represent a first step to directly examine the function of p210 in the cell. Preliminary data from this type of functional study using SLO-permeabilized cells have revealed that RM immunoglobulins block nocodazole-induced fragmentation of the GA, possibly by stabilizing membrane tubules connecting different Golgi stacks. In addition, p210 was found to be enriched in preparations of Taxol-induced microtubules from KE37 and HeLa cells (Rios, R. M., and M. Bornens, unpublished results). The possibility of a direct interaction of p210 with microtubules is now being investigated. This would well agree with the fact that this protein is peripheral. Moreover, together with the behavior of p210 during BFA treatment, this fits nicely with the known involvement of the intermediate compartment and microtubules in the biogenesis of the Golgi complex after BFA treatment.

We have shown that p210 cycles between the CGN and the intermediate compartment by effect of BFA. Does p210 cycle similarly under normal conditions? Although more experiments are necessary, our preliminary data showing localization of p210 in tubules that emanate from the Golgi complex at 16°C would suggest that it does. In that case, it must be rapidly recycled to the CGN since we have been unable to detect it in the intermediate compartment. Therefore, the intermediate compartment could serve to recycle not only proteins moving out from ER but also from the GA to their original locations.

Three other autoantigens have been previously reported to be associated with the Sjögren's syndrome: a component of ribonucleoprotein particles of mol wt 60,000, a 48-kD protein that associates to nascent transcript (Tan, 1991), and a p230 localized in the Golgi complex (Kooy et al., 1992). This p230 Golgi protein exhibits some characteristics similar to our p210 autoantigen, but also some marked differences: first, the p230 is associated with the *trans* cisternae of the GA, and second, BFA induces the dissociation of p230 from the Golgi complex to cytosol. A 200-kD Golgi protein has also been reported by Narula et al. (1992). This protein is not cisternae specific but accumulates to dilated rims of cisternae and on vesicles scattered in the Golgi region. Like p230, p200 redistributes in the cytoplasm upon BFA action, but both proteins differ in the kinetics of BFA action.

In conclusion, p210 appears to be a novel Golgi protein which might be a useful marker in deciphering the dynamics of the ER-Golgi pathway. Morphological criteria have demonstrated that p53 and p58 cycle constitutively between the ER, the intermediate compartment, and the CGN. This dynamic behavior together with the lack of a marker for the CGN has hindered the comprehension of structural and functional relationships between these three membrane-bound compartments. Recently, a novel protein, p63, that resides in the intermediate compartment and does not cycle between ER and the CGN has been identified (Schweizer et al., 1993). Here, we have identified a protein residing in the CGN that cycles between this structure and the intermediate compartment, at least in the presence of BFA. We think that both proteins, p63 and p210, represent useful tools to clarify the intimate relationships between the intermediate compartment and the GA. In addition, p210 might be an important protein for the microtubule-dependent organization of the GA.

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