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Double Immunofluorescent Staining of α2,6 Sialyltransferase and β1,4 Galactosyltransferase in Monensin-Treated Cells: Evidence for Different Golgi Compartments?

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Abstract β1,4 galactosyl- and α2,6 sialyltransferase (gal-T EC 2 4 1 22 and sialyl-T EC 2 4 99.1) sequentially elongate and terminate complex N-glycan chains of glycoproteins Both enzymes reside in *trans* Golgi cisternae, their ultrastructural relationship, however, is unknown To delineate their respective Golgi compartment(s) we conducted a double label immunofluorescent study by conventional and confocal laser scanning microscopy in HepG2, HeLa, and other cells in presence of Golgi-disturbing agents Polyclonal, peptide-specific antibodies to human sialyl-T expressed as a β-galactosidase-sialyl-T fusion protein in *E coli* were developed and applied together with mABs to human milk gal-T

In untreated HepG2 and HeLa cells Golgi morphology identified by immunofluorescent labeling of sialyl-T and gal-T, respectively, was nearly identical Treatment of cells with brefeldin A (BFA) led to rapid and coordinated disappearance of immunostaining of both enzymes, after BFA washout, vesicular structures reappeared which first stained for gal-T followed by sialyl-T, in the reassembled Golgi apparatus sialyl-T and gal-T were co-localized again. In contrast, monensin treatment produced a reversible swelling and scattering of gal-T positive Golgi elements while sialyl-T positive structures showed little change Treatment with nocodazole led to dispersal of Golgi elements in which gal-T and sialyl-T remained co-localized Treatment with chloroquine affected Golgi structure less than monensin and led to condensation of gal-T positive and to slight enlargement of sialyl-T positive structures

Sequential recovery from BFA of gal-T and sialyl-T and their segregation by monensin suggest that these enzymes are targeted to different Golgi subcompartments

Key words: brefeldin A, monensin, nocodazole, chloroquine, confocal laser scanning fluorescence microscopy, fusion protein

Much progress has been made in understanding the molecular organization of the Golgi apparatus [for reviews see Rothman and Orci, 1992; Mellman and Simons, 1992]. It has become widely accepted that intracellular traffic through the Golgi cisternal stack occurs by vesicular transport. This concept strongly relies on the assumption that individual cisternae contain their specific equipment of enzymes, glycosyltransferases in particular. Evidence in favor of their cisternal organization was first provided for galactosyltransferase (gal-T) [Roth and Berger, 1982], a glycosyltransferase involved in peripheral N- and O-glycosylation [Strous, 1986]. Localization of gal-T to *trans* cisternae was soon followed by analogous findings for N-acetylglucosaminyl-transferase I (GlcNAc-T) which was located to the medial cisternae in liver cells [Dunphy et al., 1985], a key finding to substantiate vesicular transport [Rothman and Orci, 1992]. In addition, sialyltransferase (sialyl-T) which terminates the complex N-glycan chains elongated by gal-T and GlcNAc-T has been localized to the extensive *trans* Golgi network (TGN) but also to the *trans* cisternae [Roth et al., 1985] in liver cells. Recently, in a double label immunoelectron microscopical study for gal-T and a *myc*-epitope tagged GlcNAc-T, these findings have been confirmed and extended to the notion of predominant localization rather than abso-

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lute confinement to specific cisternae [Nilsson et al., 1992].

Evidence to show that Golgi subcompartments are different biochemical entities was also obtained from attempts to dissect the Golgi apparatus by perturbing agents, notably monensin [Tartakoff, 1983a,b; Griffiths et al., 1983] and brefeldin A (BFA) [Alcalde et al., 1992]. While Griffiths et al. [1983] located a monensin-induced transport block of viral membrane proteins between medial and *trans* cisternae, immuno-electronmicroscopical evidence obtained by Strous et al. [1985] showed that mainly gal-T containing (*trans*) Golgi cisternae react to short monensin treatment by swelling. Reversible swelling of gal-T containing Golgi subcompartments is also easily shown at the level of the light microscope by immunofluorescence using specific antibodies to gal-T [Strous, 1986; our unpublished results]. Is the early monensin effect confined to Golgi subcompartments containing gal-T? If specific antibodies to other resident Golgi proteins are available, sensitivity to monensin would provide a simple assay to test for co-localization of these antigens by double immunofluorescence microscopy. The validity of this approach, however, is based on the assumption that the functional identity of the perturbed compartment remains intact. This also implies that neither membrane budding nor fusion of affected cisternal membranes would be triggered or impeded by monensin. BFA effects also have been instrumental in obtaining some information on the cisternal organization of the Golgi stack: Alcalde et al. [1992] showed by a similar approach as presented here that *cis*/medial Golgi markers reappeared before *trans* markers from BFA-induced fusion with the ER.

We propose to use this approach to test for co-localization of gal-T and sialyl-T. These enzymes add galactose and sialic acid sequentially to construct the complex outer chains of glycoproteins [Kornfeld and Kornfeld, 1985]. Previous attempts to localize sialyl-T and gal-T by double immunofluorescence yielded conflicting results: while in some cell types only a partial overlap of gal-T- and sialyl-T-positive structures was found [Berger and Hesford, 1985; Berger et al., 1987b], co-localization of both enzymes was reported in liver parenchymal cells [Taatjes et al., 1987]. The reasons for this discrepancy remained unclear but may have been related to carbohydrate specificities present in the polyclonal antiserum raised against bovine colostrum sialyl-T [Berger and Hesford, 1985]. To rule out interference by glycan antigens, polyclonal antibodies to the human sialyl-T polypeptide expressed in *E. coli* as a hybrid β-galactosidase-sialyl-T fusion protein have been newly developed and applied together with mABs to human milk gal-T [Berger et al., 1986]. Both enzymes were localized by double immunofluorescence and confocal laser scanning microscopy in undisturbed and drug-treated cells. Various agents known to specifically disturb Golgi morphology such as BFA [Lippincott-Schwartz et al., 1990; for review see Klausner et al. 1992], chloroquine [Thorens and Vassalli, 1986], and nocodazole [Thyberg and Moskalewski, 1985] have been included: These experiments demonstrate that gal-T and sialyl-T reside in Golgi subcompartments that respond differently to monensin and BFA washout.

MATERIALS AND METHODS

Biological Materials

Cell lines. HepG2 cells were obtained from the ATCC; HeLa cells were from Dr. Wunderli, Institute of Immunology and Virology, University of Zurich, human fibroblasts from Prof. Wiesmann, University of Berne, and Caco2 cells from Dr. Hauri, University of Basel. All cell lines except Caco2 were grown in DMEM supplemented with 10% fetal calf serum (Gibco); 20% FCS was used for Caco2 cells. Cells were periodically tested for absence of contamination with mycoplasma using the DAPI (Boehringer) stain.

Bacterial strain. *E. coli* strain XL1 was from Stratagene; expression vector was pTRB2 [Bürglin and de Robertis, 1987].

Oligonucleotide primers were from Microsynth (CH-5200 Windisch, Switzerland).

Antibodies to gal-T. mAB GT2/36/118 belonged to IgG3 subclass as previously described [Berger et al., 1986]. Affinity purified peptide-specific antibody to a gal-T β-galactosidase fusion protein was characterized recently [Watzele et al., 1991]. Secondary antibodies were as follows: goat anti-mouse Ig-peroxidase from Cappel; goat anti-rabbit IgG-peroxidase from Bio-Rad; goat anti-mouse IgG-TRITC from Southern Biotechnology Associates; swine anti-rabbit IgG-FITC and goat anti-mouse IgG-FITC from Nordic; goat anti-rabbit IgG-TRITC from Bioscience Products.

TABLE I. PCR Primers

Primer	Sequence (5′ to 3′)	bp in placental sialyl-T cDNA[a]
	PstI/EcoRI	
SIA1	*cgctgcagaattcc*aaa**ATG**ATTCACACCAACCTGAAGAAAAGT	1–28
	BamHI	
SIA3	*cgcggtaCC*TGTGC**TTA**GCAGTGAATGGTCCGGAAGCC	1228–1198

[a]Sialyl-T cDNA sequence from human placenta according to Grundmann et al. [1990], as published in EMBL Data Bank (Accession Nr. X17247). Capital letters represent sequences from sialyl-T; small letters are additional sequences with sites for restriction enzymes (bold). Codons for "start" and "stop" for protein synthesis are indicated.

Expression of β-Galactosidase-Sialyl-T Fusion Protein

Synthesis of sialyl-T cDNA by polymerase chain reaction (PCR). As a template to synthesize sialyl-T cDNA by PCR, 2.54 mg total RNA was isolated from a 23 × 23 cm subconfluent HepG2 monolayer according to MacDonald et al. [1987] and purified on an oligo-dT column to isolate the polyA$^+$ fraction whose quality was checked by Northern analysis using a full-length, end-labeled cDNA probe of gal-T according to standard techniques. A single band of 5.1. kb indicating nondegraded RNA was identified and found to be slightly larger than bovine mRNA coding for gal-T [Shaper et al., 1986].

To prime the PCR reaction, a 44mer oligonucleotide designated SIA1 was synthesized to contain at the 5′ end a *Pst* I and *EcoRI* restriction site linked by AAA to 28 bases of the 5′ coding region; another 38mer oligonucleotide designated SIA3 was made complementary to the last 7 amino acids preceded on the 5′ side by 7 bases of the 3′ untranslated part and a *BamHI* restriction site (see Table I). Sequence information to design SIA1 and SIA3 was from Grundmann et al. [1990]. The first cDNA strand for the PCR reaction was synthesized using Moloney Murine Leukemia Virus RNase H$^-$ Reverse Transcriptase (BRL) according to standard techniques (BRL) [Erlich, 1989]. The resulting DNA for sialyl-T had the expected size of 1,220 bp compatible with the open reading frame of a full-length cDNA of 2,6 sialyltransferase. The sialyl-T cDNA was then cloned into the *PstI* and *BamHI* sites of plasmid pIC20H. Transformation of competent DH5α cells was carried out according to standard techniques. Plasmid-DNA was isolated and analysed by restriction mapping using *HindIII* or *EcoRI* digestion. Since no *HindIII* site occurs within the cloned insert, the size of the cloned cDNA corresponded to the undigested form of 1,220 bp *EcoRI* digestion produced 2 fragments of 1,091 bp and 137 bp, respectively, compatible with the unique *EcoRI* site 129 bp downstream of the initiation codon.

Sequencing of Sialyl-T-cDNA

The inserted double strand cDNA in pIC20H was sequenced by the dideoxy method [Sanger, 1981] using an automatic sequencing device (Applied Biosystems) by using primers as for the PCR reaction, and in addition 2 oligonucleotides matching internal sequences.

Production and Purification of Sialyl-T Fusion Protein

Double digestion using *HindIII/PstI* was used to subclone the fragment into the pTRB2 expression vector [Bürglin and de Robertis, 1987] to produce a β-galactosidase sialyltransferase fusion protein (sialyl-T-FP) in *E. coli*. Plasmids containing the insert coding for sialyl-T-FP were transformed into *E. coli* strain XL1-blue [Bullock et al., 1987] according to standard procedures. For growth of the transformed strains and induction of the lacZ-sialyl-T-FP the protocol of Bürglin and de Robertis [1987] was followed. The induced cells were harvested and lysed by treatment with lysozyme and deoxycholate as outlined by Marston [1987]. Inclusion bodies enriched in fusion proteins were collected by centrifugation at 6,500*g* for 5 min. For further purification the inclusion bodies were extracted with 0.25% Triton X-100 in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl. The extract was cleared by centrifugation at 6,500*g* for 5 min and analyzed by SDS-PAGE on a 7.5% gel. The fusion proteins were then prepared on a preparative scale by SDS-PAGE on 3 mm thick 6% gels from which the bands were excised and soaked in water to elute the FP as described previously [Watzele et al., 1991]. Typically, 0.5 g bacterial wet weight yielded 3 mg sialyl-T-FP.

Dot-Blot Analysis

All incubations and washes were carried out at room temperature on a slowly moving shaker. Strips were blocked in 1% (w/v) non-fat dry milk powder in Tris-buffered saline (blocking buffer) for 60 min followed by 90 min incubation with suitably diluted mAb to human milk gal-T (GT2/36/118) (kept as 10× concentrated stocks) and suitably diluted affinity purified polyclonal antibodies to the sialyl-T-FP. Affinity purified antibodies were obtained on a sialyl-T-FP column and used at a protein concentration of 6.2 μg/ml as measured by the Bio-Rad method using bovine serum albumin as a standard. The second antibody was either a goat anti-mouse Ig-peroxidase 500-fold diluted in blocking buffer or goat anti-rabbit IgG-peroxidase 1,000-fold diluted in blocking buffer, incubated for 90 min. After three washes of each 15 min in blocking buffer between incubation with the first and second antibody and after the second antibody, 0.01% (w/v) chloro-naphthol and H_2O_2 0.03% in Tris-buffered saline was incubated with the nitrocellulose for 5 min. Prior to dot-blot analysis absorptions were carried out by adding 40 μl antigen solution containing 5 μg human milk gal-T to 0.5 ml of mAb GT2/36/118 for 60 min and 25 μl containing 20 μg sialyl-T-FP to 0.5 ml affinity purified anti-sialyl-T-FP antibody (6.2 μg/ml) for 60 min.

Metabolic Labeling of HepG2 Cells and Immunoprecipitation

Six 10 cm petri dishes containing subconfluent HepG2 cells were washed with 5 ml of prewarmed PBS and incubated with methionine-free medium for 20 min at 37°C. Cells were continuously labeled with 100 μCi [^{35}S]-met/ml in met-free medium for 2 h. Labeling was terminated by rinsing with prewarmed complete medium containing 3% FCS, followed by addition of ice-cold lysis buffer (1% Triton X-100 in PBS, 1 mM freshly diluted PMSF from a 100 mM stock solution in ethanol) for 45 min. The lysate was collected by a rubber policeman, homogenized by forcing through a 25G five-eighths gauge needle, and cleared by centrifugation at 14,000 rpm for 5 min in an Eppendorf desk-top centrifuge. The cell lysate was added to 1 volume "immunomix" containing 1% Triton X-100, 1% SDS, 0.5% Na-deoxycholate, and 1 mM PMSF and BSA to a final concentration of 5 mg/ml. Finally, the indicated volumes of antiserum and 100 μl of protein-A-Sepharose beads from a 30% (v/v) stock solution were added and rotated for 2 h at 4°C. The beads were sedimented by centrifugation and the pellet was washed three times in 0.5 × immunomix, three times in PBS, and once in H_2O. Immune complexes were separated from the beads by boiling in SDS-PAGE sample buffer [Laemmli, 1970]. Electrophoresis was performed using 10% polyacrylamide gels in presence of SDS followed by impregnation with EN^{3}HANCE (Du Pont), then dried and exposed to FUJI X-ray film.

Double Immunofluorescence on Cultured Cells

Cells were cultured on glass cover slips in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (20% for Caco2 cells) and grown to subconfluency. Cells were fixed in 3% (v/w) p-formaldehyde in PBS for 10 min at room temperature (as all subsequent steps) followed by three washes with PBS and quenching with 20 mM glycine in PBS for 10 min. A protocol for immunofluorescence similar to the one described by Berger et al. [1987b] was used. Briefly, after three washes with PBS, 0.1% saponin in PBS was added for 30 min to permeabilize the cells; saponin was then added to all reagents and wash solutions to prevent resealing. The permeabilized cells were incubated for 30 min with 25 μl of first antibody (mAb to human gal-T GT2/36/118) mixed with rabbit affinity purified antibody to human sialyl-T-FP, (protein concentration 62 μg/ml). After three washes with PBS, a mixture of second antibodies containing goat anti-mouse-IgG-TRITC and swine anti-rabbit-Ig-FITC or goat anti-mouse IgG-FITC with goat anti-rabbit IgG-TRITC was incubated for 30 min followed by three washes with PBS. The coverslip cultures were mounted on glass slides with Mowiol-propyl-gallate for microscopy [Berger et al., 1987b].

Conventional microscopy. Fluorescence was observed in a Reichert microscope equipped for epifluorescence using a long-range filter for TRITC (transparent for wavelengths ≥ 590 nm) and a restricted filter for FITC for wavelengths 520–560 nm. Thus, no signal leakage of FITC in the TRITC filter and vice versa was observed. Omission of one of the first antibodies followed by incubation with both second antibodies led to selective abolition of the corresponding FITC or TRITC stainings, respectively. Absorption of first antibodies with corresponding purified antigen

completely abolished the specific fluorescence (not shown).

Confocal laser scanning immunofluorescence. This technique was used to demonstrate co-localization. The specimens were examined with dual fluorescence mode for TRITC and FITC with a Bio-Rad MRC-500 confocal laser scanning microscope system attached to a Zeiss Axioplan microscope. Pictures were printed after normalization of the grey scale histogram with a Sony Mavigraph UP-500P [Schweizer et al., 1988].

RESULTS

Characterization of Antibodies

While mAbs to human galactosyltransferase have been characterized previously [Berger et al., 1986, 1987a], the polyclonal affinity purified antibodies have been newly developed by following the approach published recently [Watzele et al., 1991]. Since glycosyltransferases are resident proteins of the secretory pathway they are likely to be glycosylated themselves; thus, polyclonal antibodies against the native glycosyltransferases are expected to react with carbohydrate epitopes, thereby rendering them unsuitable for immunocyto- and histochemistry [Feizi and Childs, 1987]. To obtain peptide-specific antibodies, sialyl-T was expressed as a β-galactosidase fusion protein in *E. coli* which was purified as described above and used to elicit an antiserum designated SILA. The evidence that the fusion protein comprises authentic full-length sialyl-T relies on the following: the sequence of the cDNA (derived from HepG2 cells) was identical with the published sequence coding for sialyl-T which was based on a human placenta lambda GT10 library (Grundmann et al., 1990); expression of the full-length cDNA in *Saccharomyces cerevisiae* produced an enzyme activity able to incorporate NeuAc from CMP-NeuAc into asialofetuin [S. Ivanov et al., manuscript in preparation]; and, finally, the SILA antiserum crossreacted weakly with purified rat liver sialyl-T (not shown). Presence of sialyl-T in HepG2 cells was ascertained by metabolic labeling with [^{35}S]-met, followed by lysis, immunoprecipitation using SILA, SDS-PAGE/fluorography. Figure 1a shows the presence of a 44 kDa protein (SILA 5 or 10 μl) which was abolished by addition of fusion protein prior to the immunoprecipitation (SILA abs 10 μl) and which was not precipitable using preimmune serum (PIS 5 or 10 μl). The 44 kDa protein corresponds to a

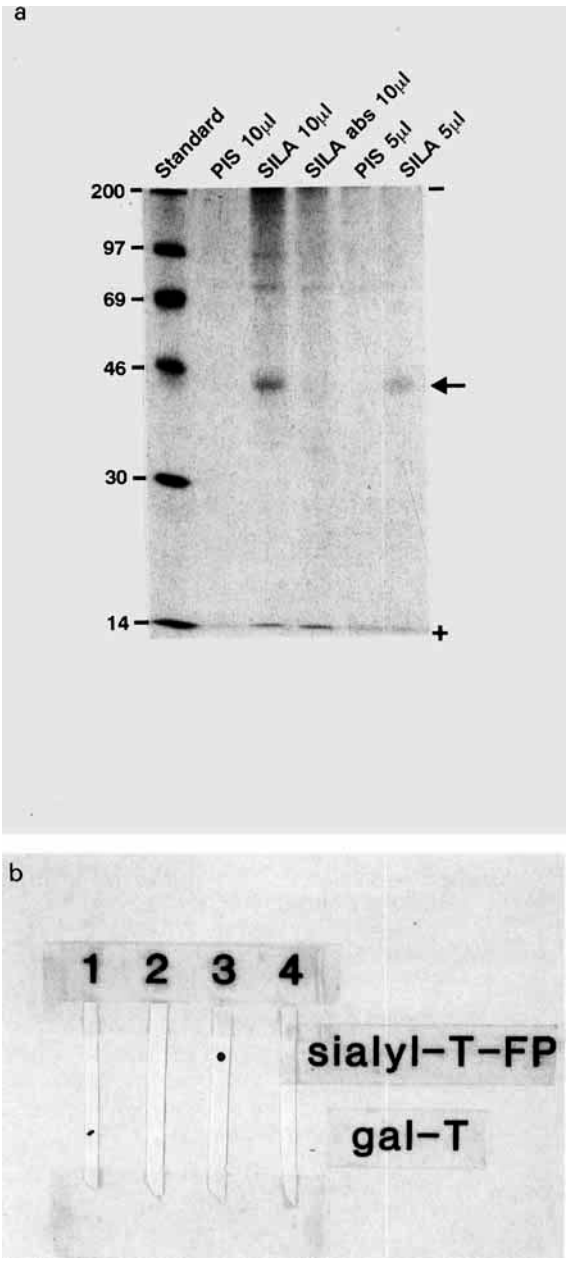

Fig. 1. Characterization of antibodies. **a:** Immunoprecipitation of sialyl-T from HepG2 cells. HepG2 cells were metabolically labeled by [^{35}S]-met, lysed, and subjected to immunoprecipitation/SDS-PAGE/fluorography using the antiserum to sialyl-T-FP (SILA 10 μl or 5 μl), the corresponding preimmune serum (PIS 10 μl or 5 μl), and antiserum preabsorbed with 20 μg sialyl-T-FP (SILA abs 10 μl). Standards are shown on the left. **b:** Dot-blot analysis of primary antibodies used for double immunofluorescence. One microliter containing approximately 1 μg of antigen was spotted on nitrocellulose strips as shown in the figure. Strips 1 and 2 were incubated with mAb GT2/36/118 (anti-gal-T), strips 3 and 4 with polyclonal antiserum to sialyl-T-FP. For strips 2 and 4, the antiserum was preabsorbed with a suitable amount of antigen (see Materials and Methods for details).

size compatible with a glycosylated form of sialyl-T [our unpublished results].

To conduct the double immunofluorescence study, absence of crossreactivity of antibodies to sialyl-T and gal-T, respectively, was ascertained by dot-blot analysis using the respective purified antigens. Figure 1b (strip 1) shows reactivity of mAb GT2/36/118 to human milk gal-T and absence of crossreactivity with sialyl-T-FP. Furthermore, affinity purified polyclonal antibodies to the sialyl-T-FP did not crossreact with gal-T (Fig. 1b, strip 3). Absorption with the respective purified antigen completely abolished the dot signal (Fig. 1b, strip 2: absorption with gal-T; strip 4: absorption with sialyl-T-FP).

Thus, we conclude that the combined use of both antibodies enables simultaneous localization of gal-T and sialyl-T by double immunofluorescence. To perform the localization studies, HepG2 cells were predominantly examined since expression of α2,6 sialyl-T is most abundant in hepatocytes [Roth et al., 1985; Taatjes et al., 1987; Paulson et al., 1989] despite the fact that, typically, HepG2 cells start piling up before reaching confluency. In addition, HeLa cells were re-investigated despite their lower content in sialyl-T.

Localization of Gal-T and Sialyl-T in Undisturbed HepG2 and HeLa Cells

Application of these antibodies to double immunofluorescence confocal laser scanning fluorescence microscopy largely confirmed apparent co-localization of both enzymes in HepG2 cells in a crescent-shaped or compact juxtanuclear structure (Fig. 2a,b) typical for the Golgi apparatus in nonpolarized cells in culture; occasionally, co-localized staining was also found in tubulovesicular Golgi elements. Co-localization was also observed in other cell types with similar Golgi morphologies such as HeLa cells (Fig. 2c,d) or different Golgi morphologies such as human fibroblasts and CaCo2 cells (not shown).

Localization of Gal-T and Sialyl-T in HepG2 Cells With Disturbed Golgi Morphology

In order to further examine the respective compartment(s), we argued that by specifically disturbing Golgi morphology by pharmacological agents, localization of both enzymes to the same Golgi subcompartment would appear as congruous FITC/TRITC staining for both enzymes. Alternatively, agents known to disturb the Golgi apparatus may segregate gal-T from sialyl-T staining. A variety of agents have been described during the last 10 years which proved useful for this approach, such as BFA [Klausner et al., 1992], chloroquine [Thorens and Vassalli, 1986], monensin [Tartakoff, 1983a], and nocodazole [Thyberg and Moskalewski, 1985]. Identification of the Golgi apparatus in most of the work cited above was based on immunofluorescence of gal-T as a marker for a genuine Golgi protein. All these agents reversibly disturb Golgi morphology in a characteristic way.

Monensin

The effect of monensin on gal-T positive Golgi cisternae leading to their swelling has been described [Strous et al., 1985]. This finding was compatible with biochemical evidence for a galactosylation block of metabolically labeled μ chains [Tartakoff, 1983a]. Monensin treatment of HepG2 cells first led to a segregation of gal-T positive elements already after 30 min (Fig. 3b,g) which was followed by the appearance of small vesicles in the Golgi region after 60 min (Fig. 3h, arrowhead). By contrast, the sialyl-T positive structures remained compact (Fig. 3c). Most of gal-T positive elements appeared to be reintegrated again into the sialyl-T positive Golgi structure 30 min after removal of monensin (Fig. 3d,i); both enzymes were co-localized again 60 min after monensin washout (Fig. 3e,k), indicating reversibility of monensin effects.

Brefeldin A

Brefeldin A (BFA) effects on Golgi morphology have been thoroughly investigated [Klausner et al., 1992] and consist of fusion of Golgi with ER membranes. As shown in Figure 4, gal-T and sialyl-T remained co-distributed during the early phase (2 min BFA: Fig. 4a,f; 5 min BFA: Fig. 4b,g). After 60 min incubation with BFA, Golgi structures completely disappeared while a diffuse cytoplasmic background staining, indicative of ER, became predominant (Fig. 4c,h). Sixty minutes after BFA washout, gal-T positive structures consistently reappeared before reappearance of sialyl-T at the same spots (Fig. 4d,i). Golgi structures appeared fully restored 120 min after BFA washout (Fig. 4e,k).

Chloroquine

Treatment of HeLa cells with 1 mM chloroquine for 30 min induced a condensation of gal-T positive elements while moderate swelling

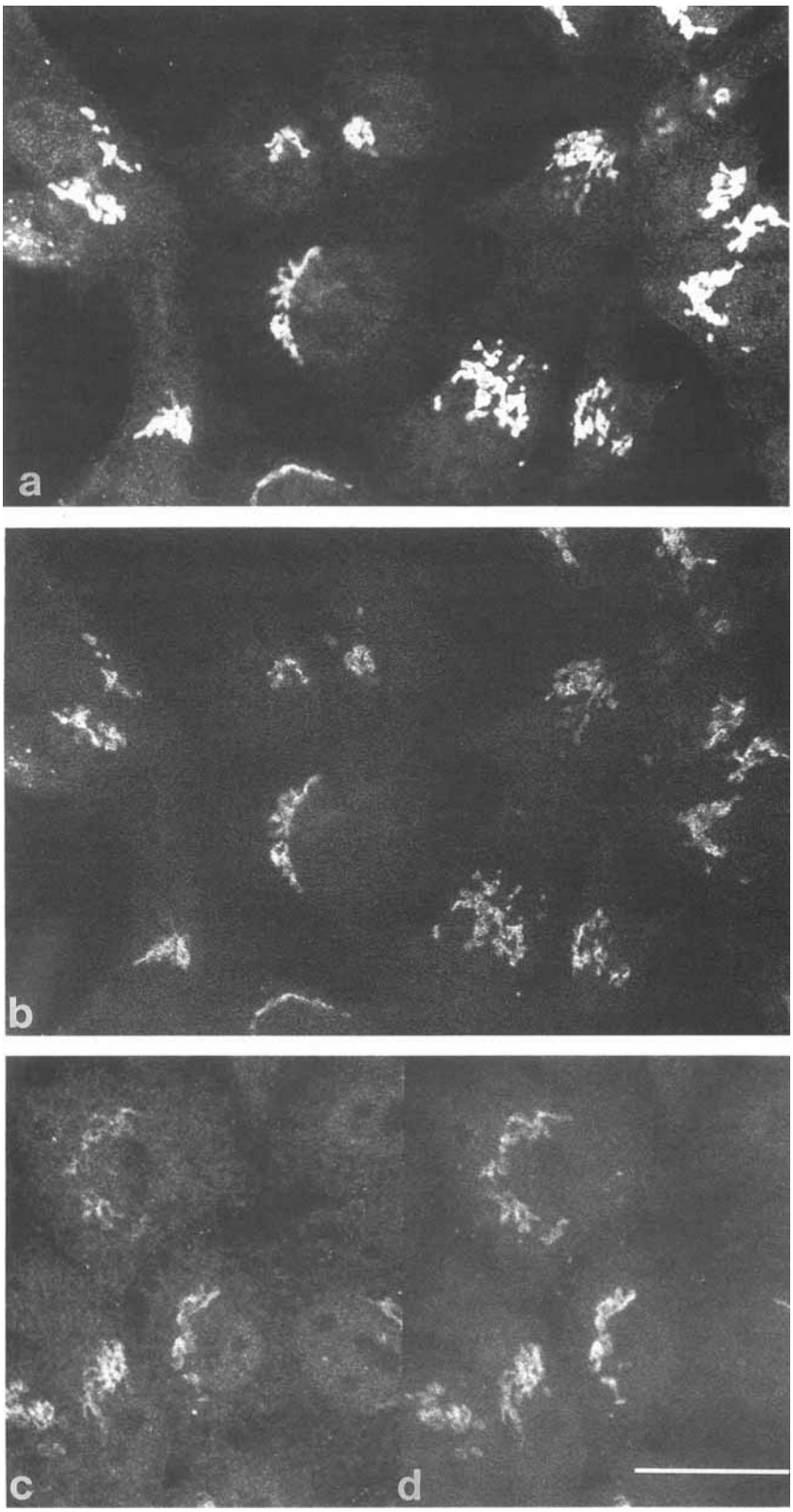

Fig. 2. Co-localization of sialyl-T (**a,c**) and gal-T (**b,d**) in HepG2 (a,b) and HeLa cells (c,d) by confocal laser scanning microscopy For details see Materials and Methods Bar, 25 μm

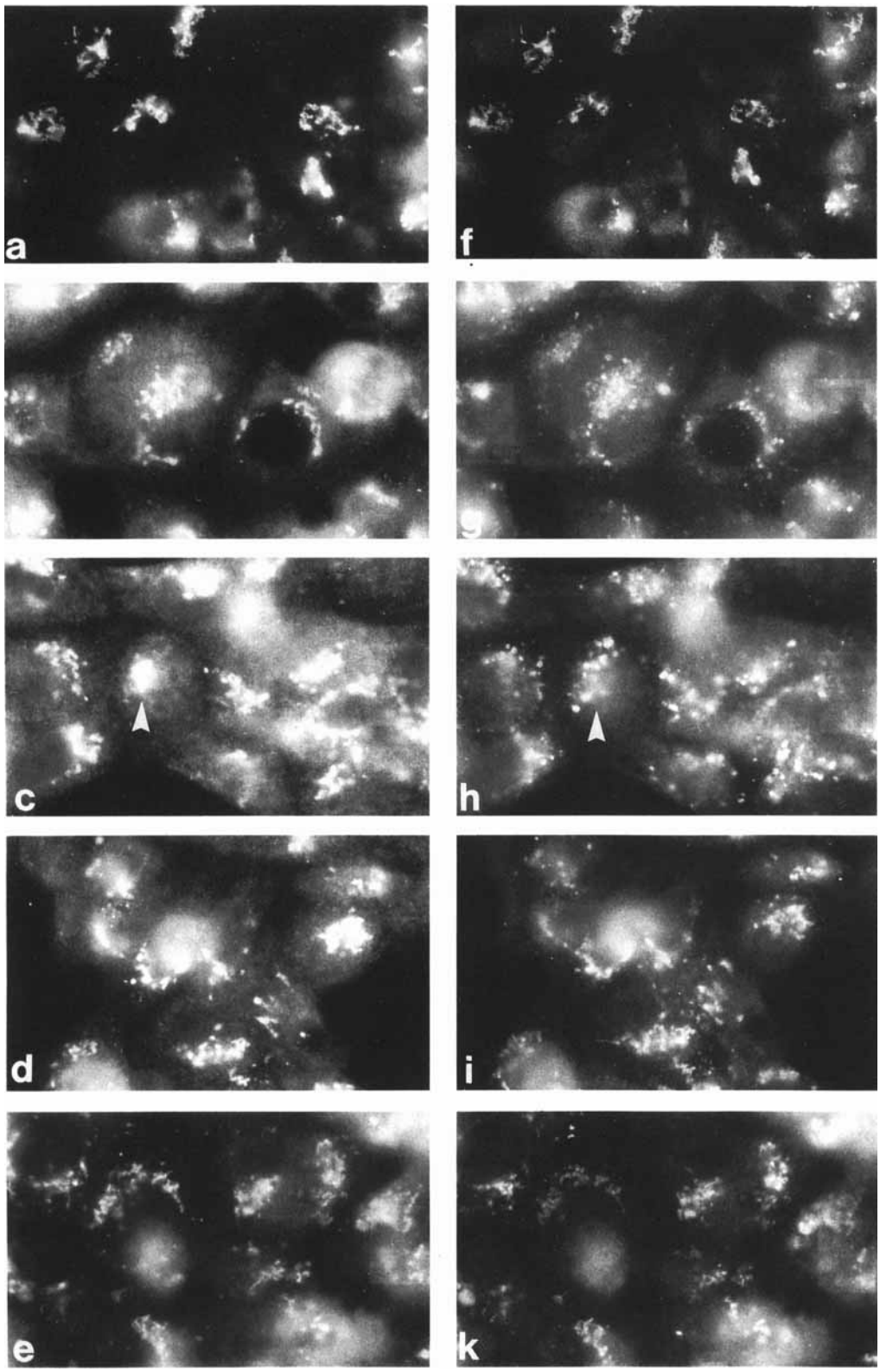

Fig. 3. Dissociation of gal-T (f–k) from sialyl-T (a–e) in Golgi elements of HepG2 cells treated with monensin **a,f:** Untreated cells **b,g:** Cells treated with 2 μM monensin for 30 min **c,h:** Arrowheads indicate scattering of gal-T (h) and maintenance of a compact sialyl-T positive structure (c) (60 min) Regeneration after incubation with monensin for 60 min followed by washout and addition of normal medium for 30 min (**d,i**) and 60 min (**e,k**).

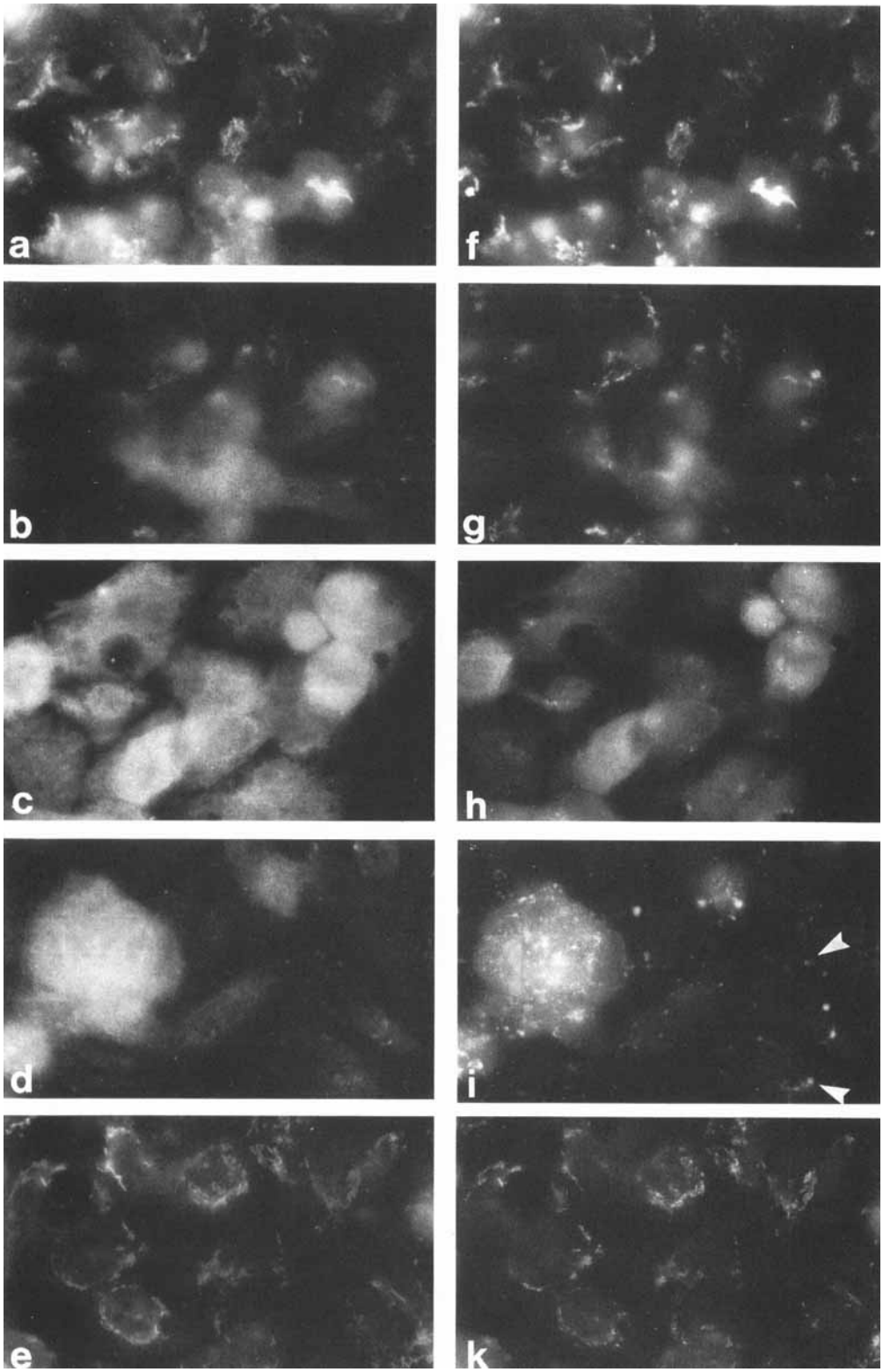

Fig 4 Effect of brefeldin A on sialyl T (a–e) and gal T (f–k) positive Golgi structures in HepG2 cells HepG2 cells were treated with 20 μg/ml BFA for 2 min (**a,f**) 5 min (**b,g**), 60 min (**c,h**), regeneration after incubation with BFA for 60 min followed by washout and addition of normal medium for 60 min (**d,i**) and 120 min (**e,k**) The arrowheads in i indicate reappearing gal T positive vesicles which are not yet visible in d

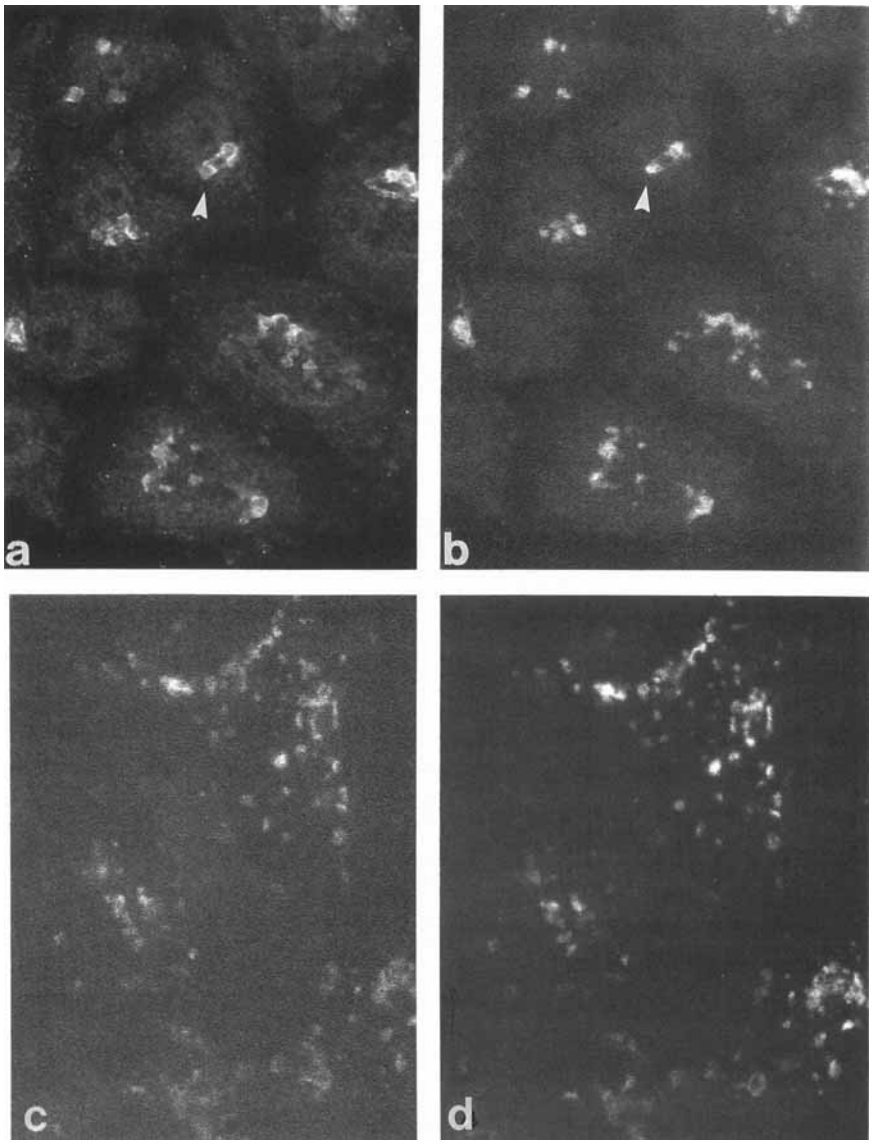

Fig. 5. Effect of chloroquine (a,b) and nocodazole (c,d) on sialyl-T (a,c) and gal-T (b,d) positive Golgi structures in Hela cells. **a,b:** Incubation with 1 mM chloroquine for 30 min. Fluorescence of sialyl-T detected by CLSM concentrates on the rims of a slightly enlarged Golgi structure (a, arrowhead); gal-T consistently appears in a condensed form (b, arrowhead). **c,d:** Incubation (60 min) with 15 μg/ml nocodazole; gal-T and sialyl-T remain co-localized in scattered Golgi elements.

of sialyl-T containing compartments was observed (Fig. 5a,b, arrowhead). Similar results were obtained in HepG2 cells (not shown).

Nocodazole

Nocodazole is a drug known to reversibly disintegrate microtubules which, in turn, leads to scattering of Golgi elements throughout the cytoplasm [Kreis, 1990]. Ultrastructural data from nocodazole-treated cells revealed intact Golgi cisternal stacks [Thyberg and Moskalewski, 1985]. Figure 5c,d shows that Golgi elements were scattered 1 h after addition of nocodazole, but that both gal-T and sialyl-T remained associated. Persistent co-localization was ascertained on different focal planes by confocal laser scanning microscopy (not shown), indicating that nocodazole treatment did not dissociate the compartments containing either gal-T or sialyl-T, respectively.

DISCUSSION

Localization and topogenesis of genuine Golgi proteins is being actively investigated by several

groups since mechanisms of their retention to Golgi membranes may reveal some basic principles of assembly of the Golgi apparatus. More specifically, two representative enzymes have recently been studied in detail: galactosyl- and sialyltransferase [for review see Hurtley, 1992]. Among the multitude of processing enzymes located in the Golgi apparatus, these examples are of interest because they act sequentially on acceptor substrates in transit through the Golgi stack: gal-T elongates glycan structures to form acceptor substrates for sialyl-T which terminates glycan biosynthesis. The main findings presented here show that Golgi disturbing agents such as monensin and BFA affect the cellular sites to which gal-T and sialyl-T are localized, in different ways. Two alternative explanations for this differential effect shall be considered: either segregation by monensin of gal-T from sialyl-T positive structures indicates the existence of different compartments or, alternatively, monensin may interfere with a putative retrieval pathway for gal-T by inhibiting fusion of gal-T positive vesicles.

Our data rely on the use of newly developed antibodies to a sialyl-T peptide expressed as a fusion protein in *E. coli* and hence devoid of carbohydrate antigens. Similarly, mAb used to localize gal-T most likely recognize the peptide as reported previously [Berger et al., 1987a]; however, since the epitope recognized by this mAB has not been investigated in detail, we also developed polyclonal antisera to a gal-T fusion protein in *E. coli* [Watzele et al., 1991] and conducted double label immunofluorescent localization with the mAb to gal-T under similar conditions as described here: we always observed co-localization of the epitopes recognized by the peptide-specific and the mAb (our unpublished results). Thus, we believe that the use of our reagents permits localization of the authentic products of the gal-T and sialyl-T genes. This is the more important as in the case of so-called ectoglycosyl-transferases, carbohydrate antigens have led to misinterpretations of immunocytochemical data [Feizi and Childs, 1987], a recent notable example being the absence of detectable ecto-sialyltransferase (Stamenkovic et al., 1991) using the antibodies as reported here on CDw75 positive cells while in the same cells the Golgi apparatus was clearly stained [Keppler et al., 1992].

Previously, we reported only partial co-localization of sialyl-T and gal-T in several cell lines [Berger and Hesford, 1985; Berger et al., 1987b] and observed a more vesicular distribution of sialyl-T in several cell lines. The antiserum we have used was raised against bovine colostrum sialyl-T and may have contained some carbohydrate specificities leading to incorrect stainings. Using the antiserum as described here, we consistently find a Golgi morphology which appears indistinguishable from the classical Golgi staining using anti-gal-T antibodies (Fig. 2). Thus, co-localization even to the same cisternae appeared likely with the proviso that only double immuno-electron microscopy for both enzymes, not reported yet, would allow for this conclusion. Much to our surprise, treatment by monensin dramatically dissociated immunoreactive staining of gal-T from sialyl-T (Fig. 3). This observation is easily explained by postulating different compartments containing (predominantly or exclusively) one of both enzymes and being differentially sensitive to monensin. As stated above, this explanation implies that the functional integrity of individual Golgi cisternae remains intact during monensin treatment. In fact, there is supporting evidence for this assumption by the observation that even 4 h after monensin treatment, cytochemical markers remain confined to individual swollen cisternae [Griffiths et al., 1983]. The immuno-electronmicroscopical evidence of gal-T associated with the membranes of swollen cisternae [Strous et al., 1985] appears also to be compatible with this interpretation. Thus, if the assumption of maintenance of the functional integrity of Golgi compartments proves true, monensin treatment followed by immunofluorescent staining for glycosyltransferases provides an easy assay for their topogenesis. An alternative explanation for the monensin effect appears also possible in the light of recent data reporting the comparative localization of gal-T and GlcNAc-T in HeLa cells: these glycosyltransferases were found in several adjacent cisternae but at different densities. Thus, some gal-T antigenic sites were found in transmost cisternae while their majority was located in *trans* cisternae. Similarly, in these latter compartments some GlcNAc-T antigenic sites were found while most of them were located in the medial cisterna [Nilsson et al., 1993]. Considering the continuous vesicular traffic across the Golgi stack, a hypothetical retrieval mechanism may operate which returns "escaped" enzyme to the compartment where it works most efficiently. The effect of monensin

could then consist in inhibition of reentry of retrieved gal-T leading to its segregation. Clearly, more work is required to validate these explanations.

No obvious difference was found between the response of gal-T and sialyl-T positive structures to BFA. This finding is not easily reconciled with data reported by Chege and Pfeffer [1990]: relocation of sialyl-T to the ER after BFA (Fig. 4) does not argue in favor of sialyl-T to be a marker for the TGN as assumed by these authors; however, kinetic differences between reassembly of different cisternal markers as reported by Alcalde et al. [1992] reflects sequential buildup of adjacent cisternae. Thus, by analogy, reappearance of gal-T before sialyl-T (Fig. 4d,i), argues in favor of different compartments. Taken together, monensin-induced segregation of these enzymes more likely indicates different compartments than a blocked retrieval pathway for gal-T.

Are our findings on Golgi perturbing drugs compatible with previous assumptions on their effects? In the case of nocodazole treatment, both enzymes remained co-distributed in Golgi-derived structures. Nocodazole is known to dissociate microtubules leading to dispersal of the Golgi apparatus; the cisternal stacks, however, remain intact [for reviews see Thyberg and Moskalewski, 1985; Kreis, 1990]. This appears compatible with a model in which "default" localization of sialyl-T is confined to the cisternal portion of the TGN. The effects of monensin and acidotropic agents such as chloroquine have been reviewed by Mellman et al. [1986]. They noted that both agents disturb Golgi cisternae by causing dilation in an "at least superficially similar" manner. At that time it was known that monensin predominantly affects gal-T positive cisternae [Strous et al., 1985] while the target compartments for chloroquine action have not been correlated with any other Golgi function. Subsequently Thorens and Vassalli [1986] provided evidence that treatment of plasma cells by chloroquine inhibited sialylation of immunoglobulins while galactosylation was still possible. This finding appeared to be in contradiction to the notion of co-distribution of both sialyl-T and gal-T established on the basis of double immunofluorescence in hepatocytes [Taatjes et al., 1987]. Our results provide a basis to render these observations compatible: while sialyl-T and gal-T appear to be co-localized in undisturbed cells at the level of light microscopy (Fig. 2), both monensin (Fig. 3) and (to a lesser extent) chloroquine (Fig. 5a,b) treatments induced a segregation of both enzymes. More interestingly, they confirmed that within the Golgi stack, chloroquine affects primarily sialyl-T and monensin the gal-T containing compartments. These results also provide a morphological basis to the observation that mannose-6-phosphate receptors recycle through Golgi compartments in which sialylation but not galactosylation occurs [Duncan and Kornfeld, 1988].

The molecular mechanisms causing the morphological alterations of the Golgi apparatus are believed to rely on facilitating electroneutral exchange of Na^+, K^+, and H^+ in the case of monensin and the dissipation of transmembrane pH gradients by chloroquine [Mellman et al., 1986]. Although this interesting aspect of the heterogeneous response of Golgi cisternal membranes to these drugs is not the focus of this study, our data indicate that in addition to their biosynthetic differences, cisternal membranes exhibit also differences in ion transport mechanisms. Thus, both enzymatic and transport characteristics may contribute to the unique properties of individual cisternae; this may be relevant to maintain the proper ionic environment for glycosylation [Tartakoff, 1983b]. Thus, ion transport defects may cause pleiotropic changes in glycosylation as recently reported for sialylation in cells affected by cystic fibrosis [Barasch et al., 1991].

In summary, the use of Golgi perturbing agents provided new insight into the functional organization of terminal glycosylation in the Golgi apparatus: segregation of gal-T from sialyl-T by monensin more likely indicates different compartments for both enzymes; however, interference with a retrieval pathway of gal-T by monensin cannot be ruled out.

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