Isolation of a Matrix That Binds *Medial* Golgi Enzymes

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Abstract. Rat liver Golgi stacks were extracted with Triton X-100 at neutral pH. After centrifugation the low speed pellet contained two *medial*-Golgi enzymes, *N*-acetylglucosaminyltransferase I and mannosidase II, but no enzymes or markers from other parts of the Golgi apparatus. Both were present in the same structures which appeared, by electron microscopy, to be small remnants of cisternal membranes. The enzymes could be removed by treatment with low salt, leaving behind a salt pellet, which we term the matrix. Removal of salt caused specific re-binding of both enzymes to the matrix, with an apparent dissociation constant of 3 nM for mannosidase II. Re-binding was abolished by pretreatment of intact Golgi stacks with proteinase K, suggesting that the matrix was present between the cisternae.

A stack of closely apposed and flattened cisternae is the central feature of the Golgi stack (Farquhar and Palade, 1981). It resists unstacking during purification (Morré and Mollenhauer, 1964; Morré et al., 1970), suggesting that there are links between adjacent cisternae and these links must be numerous because the width of the intercisternal space is relatively constant (Cluett and Brown, 1992). The most likely candidates for forming such links are the cytoplasmic tails of resident enzymes, such as those involved in modifying N-linked oligosaccharides. These are reasonably abundant proteins, constituting a few percent of each cisternal membrane, and are present throughout the Golgi stack, their exact cisternal position varying from enzyme to enzyme and between different cell lines (Roth, 1987). A number of these proteins has been cloned and sequenced (Weinstein et al., 1987; Kumar et al., 1990; Watzele and Berger, 1990; Moremen and Robbins, 1991). The cytoplasmic tails have been found to vary in length from 5 to 24 amino acids, and no obvious consensus has been found. The longest could only span a distance of 36 Å as an α-helix, which is less than half the width of the intercisternal space (100 Å; Cluett and Brown, 1992). Additional intercisternal proteins would, therefore, be needed to link these cytoplasmic tails.

Components of an intercisternal matrix have been visualized by several workers in a variety of systems and appear either as inter-cisternal elements of electron-dense material running between and parallel to the cisternae (Amos and Grimstone, 1968; Mollenhauer, 1965; Mollenhauer et al., 1973; Mollenhauer and Morré, 1975), or as regularly spaced intercisternal cross-bridges (Franke et al., 1972). Such cross-bridges were also found to connect membranes of several organelles such as the endoplasmic reticulum (ER) and the thylakoid membranes of the chloroplast (Franke et al., 1971). Isolated Golgi stacks contain similar cross-bridges which are sensitive to treatment with a variety of proteases (Cluett and Brown, 1992). Such treatment also causes the cisternae to separate which implicates the cross-bridges in the stacking mechanism.

As an approach to the biochemical isolation of components of an intercisternal matrix, we have drawn upon the procedures used for the characterization of the nuclear lamina. The lamina is a meshwork of intermediate filaments (Aebi et al., 1986) linking chromatin to proteins in the inner nuclear envelope membrane (Worman et al., 1988). It was isolated as a rapidly sedimenting structure resistant to extraction with Triton X-100 (TX-100)[1] and low salt (Dwyer and Blobel, 1976). By applying very similar procedures to highly purified rat liver Golgi stacks, we have isolated a matrix that binds only *medial*-Golgi enzymes and does so with high affinity.

Materials and Methods

Materials

All reagents were of analytical grade or higher and purchased from either Sigma Chemical Co. (St. Louis, MO) or BDH Ltd. (Poole, England), except for radiochemicals (NEN, Boston, MA), unless otherwise stated.

Preparation of Rat Liver Golgi Stacks

Fresh livers were homogenized by passage through a 150-μm mesh sieve (Hino et al., 1978) into ice-cold 0.5 M sucrose-PM buffer (0.1 M sodium phosphate, pH 6.7, 5 mM $MgCl_2$) to 0.6 g liver/ml buffer. This was applied to a two-step gradient comprising 1.3 and 0.86 M sucrose-PM buffer. After centrifugation at 28,000 rpm using a SW28.Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) for 1 h at 4°C, the Golgi fraction was collected from

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1. *Abbreviations used in this paper*: GalT, β1,4-galactosyltransferase; Mann I, α1,2-mannosidase I; Mann II, α1,3-1,6-mannosidase II; NAGT I, β1,2-*N*-acetylglucosaminyltransferase I; TX-100, Triton X-100.

the 0.5/0.86 M sucrose interface. After dilution to 0.25 M sucrose using PM buffer, the membranes were pelleted at 7,000 rpm for 30 min at 4°C, washed in 0.25 M sucrose-PM buffer, re-pelleted and stored at −80°C in the same buffer. The purification over homogenate was typically 80-fold for (β1,4-galactosyltransferase) GalT and 150-fold for β1,2-*N*-acetylglucosaminyltransferase I (NAGT I). All of the NAGT I is present in stacked cisternae (Dunphy et al., 1985; Nilsson et al., 1993*a*) whereas a large part of the GalT is present in the *trans*-Golgi network (Nilsson et al., 1993*a*), which tends to be partially lost in these preparations. This would explain the different purification factors.

Extraction with TX-100

Rat liver Golgi stacks were recovered by centrifugation at 50,000 rpm for 5 min at 4°C in a TL-100 centrifuge (Beckman Instruments, Inc.) using a TLA-100.1 rotor, and resuspended in TMMDS buffer (2% [wt/vol] TX-100, 50 mM MOPS-NaOH, pH 7.0, 0.1 mM $MgCl_2$, 1 mM DTT, 10% [wt/vol] sucrose) to 1 mg of protein/ml as determined by using the BCA Protein Assay Kit (Pierce, Rockford, IL). The suspension was incubated on ice for 30 min and then centrifuged at 20,000 rpm for 30 min at 4°C. The supernatant was removed and the pellet washed gently in 2 volumes of TMMDS buffer before careful resuspension in one volume of the same buffer.

SDS-PAGE

SDS-PAGE was carried out as described by Blobel and Dobberstein (1975) after precipitation of protein by the method of Wessel and Flugge (1984). Protein was visualized using Coomassie brilliant blue R.

Microsequencing

After SDS-PAGE proteins were transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA) using a semi-dry blotter and a 10-mM CAPS, pH 11/2% (vol/vol) methanol blotting buffer. Proteins were visualized with sulpho-rhodamine B (Coull and Pappin, 1990) and sequenced on a MilliGen 6600 solid phase protein sequencer (MilliGen, division of Millipore, Bedford, MA).

Assays for Golgi Markers

α1,2-Mannosidase I (Mann I; EC 3.2.1.113). This was assayed as described by Tabas and Kornfeld (1979) except that separation of released [^{3}H]-mannose from substrate and trace complex oligosaccharides was achieved by filtration through a 0.5-ml, mixed (1:1) Concanavalin A–Sepharose and wheat germ agglutinin–agarose column. The substrate was prepared from spinner Hela cells incubated in low glucose DME for 24 h with 5 μCi/ml [^{3}H]mannose in the presence of 20 μg/ml deoxy-mannojirimycin (dMM; Boehringer Mannheim, Mannheim, Germany). dMM specifically blocks mannosidase I (Bischoff and Kornfeld, 1984) so that the N-linked oligosaccharides bound to transported proteins are only processed as far as the $Mann_{7-8}$-$GlcNAc_2$ stage. These substrate molecules were released by treatment with proteinase K after extraction of integral-membrane proteins with Triton X-114 as described by Featherstone et al. (1985).

β1,2-N-Acetylglucosaminyltransferase I (NAGT I; EC 2.4.1.101). This was assayed as described by Vischer and Hughes (1981) using ovalbumin as an acceptor and an incubation time of 2.5 h. Precipitated pellets were washed once with 1% (wt/vol) phosphotungstic acid/0.5 M HCl and once with ice-cold 95% ethanol before resuspension in unbuffered 0.4 M Tris/4% (wt/vol) SDS for scintillation counting.

α1,3-1,6-Mannosidase II (Mann II; EC 3.2.1.114). This was assayed spectrophotometrically as described by Bischoff and Kornfeld (1984) except that the concentration of the substrate, *p*-nitrophenyl-α-D-mannopyranoside, was 2 mM in 100 mM sodium phosphate, pH 6.0. The assay was carried out for 1 h.

β1,4-Galactosyltransferase (GalT; EC 2.4.1.22). This was assayed essentially as by Bretz and Staubli (1977) using ovomucoid as an acceptor and a 30-min incubation time. Precipitated pellets were processed as for the NAGT I Assay.

TGN38 and p58. After SDS-PAGE, proteins were transferred onto Hybond-C Super membranes (Amersham International, Buckinghamshire, England) using a semi-dry blotter in a transfer buffer of 20 mM Tris, 150 mM glycine, 20% (vol/vol) methanol and 0.5% (wt/vol) SDS. Filters were blocked overnight in 5% (wt/vol) non-fat milk powder and 0.2% (wt/vol) Tween-20 in PBS and probed using affinity-purified rabbit polyclonal antibodies to TGN38 (Luzio et al., 1990) or p58 (Saraste and Svensson, 1991), followed by a secondary antibody conjugated to horseradish peroxidase (HRP; Tago Inc., Burlingame, CA). Unbound antibodies were removed by two successive 1-h washes in blocking buffer. Bound HRP was visualized using the ECL detection system (Amersham International) and quantitated using an Ultroscan XL laser densitometer (LKB).

ER and Lysosomal Marker Assays

Microsomal NADH-cytochrome c reductase activity was assayed as described by Sottocasa et al. (1967) in the presence of 1.5 μM rotenone to inhibit the mitochondrial activity. Reaction volumes were however reduced to 1 ml and 50 μl of sample was assayed. *N*-acetyl-β-hexosaminidase (EC 3.2.1.30) was measured as described by Landegren (1984).

Immuno-Isolation on Magnetic Beads

This was performed as described by Howell et al. (1989). Secondary antibodies, either 53FC3 (Burke et al., 1982) or OKT8 (ATCC CRL8014), were conjugated to magnetic-beads coated with goat anti-mouse IgG (Dynal Inc., Great Neck, NY) by rotation for 16 h at 4°C (1 μl ascites/2 μl beads), and washed 5 times with 1 ml PBS containing 5 mg/ml BSA with 15-min incubations between each wash.

The Triton pellet after Golgi extraction was washed twice in MMDS buffer and twice with PBS/BSA before resuspension to 1 mg original Golgi protein/ml. Increasing amounts of this suspension (50–500 μl) were added to 20 μl of packed, coated beads and made up to 1 ml with PBS/BSA. These samples were rotated for 2 h at 4°C and washed as above before resuspension in 100 μl of PBS/BSA. These beads were either processed for electron microscopy or assayed for enzyme content.

Electron Microscopy

Samples were fixed at 4°C overnight in 0.1 M sodium phosphate, pH 6.7, 0.25 M sucrose, 5 mM $MgCl_2$ and 0.5% (vol/vol) glutaraldehyde (Fluka Chemie AG, Buchs, Switzerland) before processing as described by Pypaert et al. (1991). Sections were examined using a Philips CM10 transmission microscope (Philips Technologies, Cheshire, CT).

Stereology

At least seven randomly selected, low magnification micrographs were used for the quantitation of Golgi stacking. A Golgi cisterna was defined as being a membrane profile whose length was at least four times its width. Stacked profiles overlapped by at least half their length with a gap between them that was less than half their width.

Salt Extraction and Reconstitution

The Triton pellet after Golgi extraction was re-extracted and centrifuged exactly as before using TMMDS buffer supplemented with NaCl at the concentrations indicated in the text and figure legends. Alternatively, Golgi stacks were extracted simultaneously with both TX-100 and salt using TMMDS containing 150 mM NaCl.

Dialyses were performed using a System 100 micro-dialyzer unit (Pierce) fitted with a Spectrapor membrane with a 12–14-kD cut-off (Spectrum Medical Industries, Inc., Houston, TX). 100-μl samples were dialyzed for 16 h at 4°C against TMMS buffer flowing through continuously at a rate of 0.3 ml/min.

After dialysis, samples were removed from the dialyzer and spun at 20,000 rpm in a TLA-100.1 rotor for 30 min at 4°C. Supernatants were removed and the pellets washed in two volumes and resuspended in an equal volume of TMMS before assaying each for the appropriate enzymes.

Scatchard Analysis

Golgi stacks (20–100 μg) were extracted with TMMDS followed by TMMDS/ 25 mM NaCl. These supernatants were made up to 100 μl with TMMDS and used to resuspend salt pellets derived from 25 μg of Golgi stacks extracted with TMMDS followed by TMMDS/150 mM NaCl. Mann II and NAGT I were assayed both before and after dialysis and sedimentation. Enzyme concentrations were calculated assuming a specific activity of 6.75 μmoles/min/mg for Mann II (Moremen et al., 1991), and 2.5 μmoles/min/mg for NAGT I (Oppenheimer and Hill, 1981). The bound enzyme was that in the pellet and the free enzyme was defined as the total loaded minus the amount bound.

Protease Treatment

Golgi stacks were diluted to 0.25 mg/ml in MMDS buffer and were incubated at 25°C for 30 min in the presence or absence 0.5 mg/ml proteinase K (Boehringer Mannheim). After quenching with PMSF, at a final concentration of 40 μg/ml, on ice for 10 min, Triton supernatants and pellets were prepared and assayed for NAGT I and Mann II.

Alternatively, the treated samples (from 100 μg Golgi stacks) were extracted simultaneously with both TMMDS/150 mM NaCl and dialyzed in the absence or presence of a salt pellet derived from 100 μg untreated Golgi stacks using TMMDS followed by TMMDS/150 mM NaCl.

Results

Extraction with Triton X-100

Rat liver Golgi stacks were purified using an adaptation of the method described by Leelavathi et al. (1970) which involves forcing minced liver through a sieve so as to preserve the stacked morphology of the cisternae (Hino et al., 1978). Stereological analysis showed that 65% ± 4 (±SEM, n = 3) of the cisternae in these preparations were present in stacks and the average stack contained 2.43 ± 0.03 (±SEM, n = 3) cisternae.

These membranes were extracted on ice with 2% (wt/vol) TX-100 in 50 mM MOPS-NaOH, pH 7.0, 0.1 mM $MgCl_2$, 1 mM DTT and 10% (wt/vol) sucrose. After 30 min the mixture was fractionated by centrifugation at 15,500 g_{av} for 30 min and the pellet washed twice. This pellet comprised ~40% of the total Golgi protein and contained one obviously insoluble protein of 125 kD (Fig. 1, see lanes *2* and *3*). This pellet could also be sedimented at lower speeds (5,000 g_{av} for 30 min), comparable with those required to sediment Golgi stacks indicating that the pellet proteins were contained within large structures (data not shown).

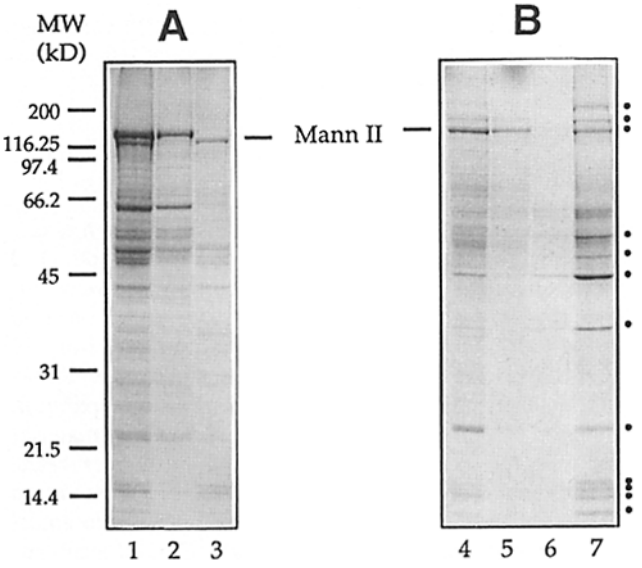

Figure 1. Extraction of Golgi stacks with Triton X-100 and salt. (*A*) Rat liver Golgi stacks (20 μg, lane *1*) were extracted with 2% TX-100 and centrifuged at low speed to give a supernatant (lane 2) and pellet (lane *3*). (*B*) A Triton pellet (from 100 μg Golgi stacks, lane *4*) was further extracted with 150 mM NaCl and centrifuged at low speed to give a supernatant (lane *5*) and pellet (lane *6*). A 5 times loading of this salt pellet is shown in lane *7*. Molecular weight markers are shown to the left and the major proteins indicated on the right are those consistently seen in the salt pellet. Proteins were fractionated by SDS-PAGE and stained with Coomassie blue R.

Chymotrypsin treatment of the 125-kD protein produced an abundant 110-kD cleavage fragment and both the intact protein and the chymotryptic fragment were blotted onto polyvinyledenedifluoride membrane for NH_2-terminal microsequencing. The sequences determined for the intact protein and the chymotryptic fragment were SXQFTVFXSAXFXVVIFXL and QADPXDXLFASQXGXQ respectively (where X = unassigned residue). Both of these sequences were identical to those for murine mannosidase II (Mann II) reported by Moremen and Robbins (1991). The difficulty in solubilizing this enzyme with TX-100 had also been reported by Tulsiani et al. (1977). We therefore conclude that this *medial*-enzyme is a major component of the Triton pellet.

Given this result the Triton pellets and supernatants were assayed for a variety of other Golgi markers: p58 (Saraste and Svensson, 1991) and mannosidase I (Mann I; Tabas and Kornfeld, 1979) were used as markers for the *cis*-Golgi network and *cis*-cisternae; *N*-acetylglucosaminyltransferase I (NAGT I; Dunphy et al., 1985; Nilsson, 1993*a*) and Mann II (Burke et al. 1982) for the *medial*-cisternae; β1,4-galactosyltransferase (GalT) for the *trans*-cisterna (Roth and Berger, 1982; Lucocq et al., 1989); and TGN38 for the *trans*-Golgi network (Luzio et al., 1990).

TGN38 and p58 were assayed by quantitative Western blotting and the other markers by enzyme assay. All of the assays were specific except that for Mann II. The substrate used for this enzyme, *p*-nitrophenyl-α-D-mannopyranoside, is also cleaved by the soluble and membrane-bound mannosidases of the ER and by lysosomal mannosidase (Bischoff and Kornfeld, 1984). Purified Golgi stacks were enriched 80-fold for GalT and 150-fold for NAGT I over the homogenate, whereas neither the ER nor lysosomes co-purified with the Golgi stacks. Lysosomes were enriched 0.75-fold using *N*-acetyl-β-hexosaminidase as the marker and the ER was enriched 0.37-fold by measuring rotenone-sensitive NADH-cytochrome c reductase activity. Furthermore, addition of swainsonine, which inhibits all mannosidases with the exception of Mann I and the ER mannosidases (Dorling et al., 1980; Tulsiani et al., 1982), abolished all of the measured activity. This confirmed the absence of ER mannosidases in the preparations of Golgi stacks and the inability of Golgi Mann I to act on the substrate (Bischoff and Kornfeld, 1984). Finally, interference by soluble ER mannosidases released during homogenization could be discounted because the Golgi stacks were washed extensively during purification.

The percentage of each Golgi marker found in the Triton pellet is presented in Fig. 2 *A*. More than 77% of NAGT I and 86% of Mann II were found in the pellet compared with only 35% of p58 and <20% of each of the other markers. These results could not be explained by selective activation or inactivation of the enzymes released into the supernatant, since the recovery of enzyme activity was close to 100% for GalT, Mann II and NAGT I (Fig. 2 *B*). Mann I was activated during the extraction and the recovery was 206%, but since the enzyme in both the supernatant and pellet was activated (data not shown), the distribution should not have been affected.

The insolubility of Mann II enzymatic activity closely matched the insolubility of the protein (Fig. 1, lanes *2* and *3*), arguing that enzymatic activity is a good measure of pro-

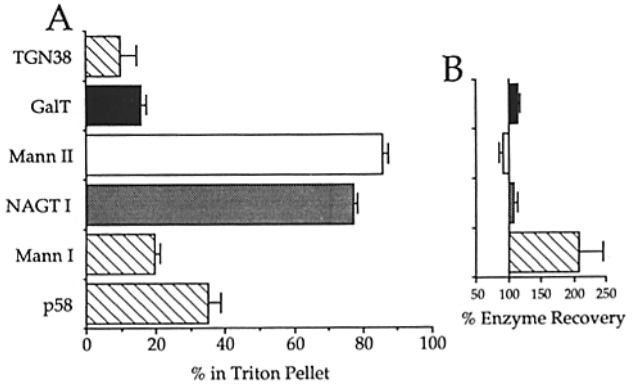

Figure 2. Golgi markers in the Triton pellet fraction. (*A*) Rat liver Golgi stacks were extracted with 2% TX-100 and centrifuged at low speed to give a pellet and supernatant fraction. Golgi markers in both fractions were assayed and the percentages in the pellet are presented ± SEM (n = 5 for Mann II, GalT and NAGT I; n = 4 for Mann I and TGN38; n = 3 for p58). Mann II and NAGT I are the only markers that are mostly present in the Triton pellet. (*B*) The percentage recoveries of the enzyme activities are presented ±SEM.

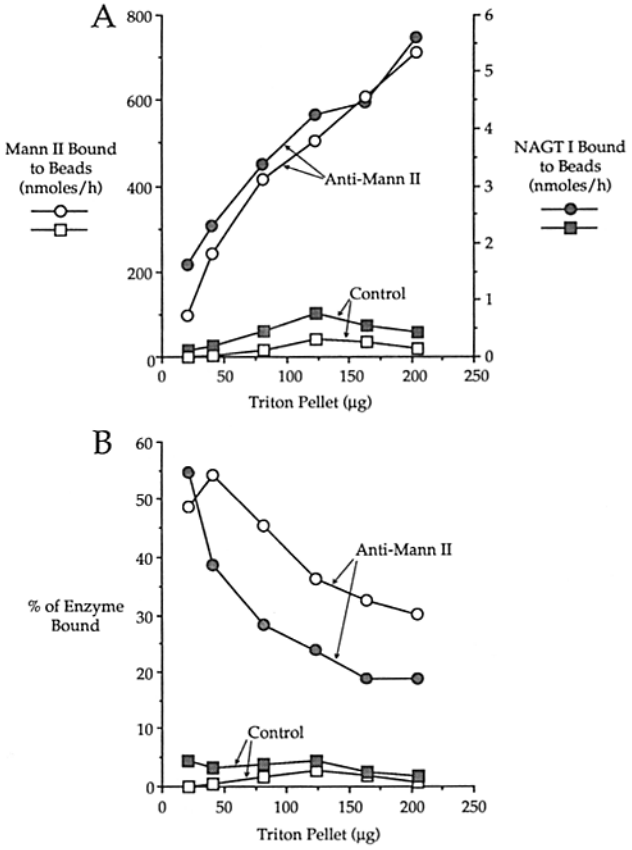

Figure 3. Immuno-isolation of Mann II in the Triton pellet fraction. Magnetic beads were coated with either control (OKT8) or anti-Mann II (53FC3) antibodies and incubated with increasing amounts of the resuspended Triton pellet. After centrifugation and washing, the beads were assayed for Mann II and NAGT I activities. (*A*) Absolute amounts of Mann II and NAGT I bound. (*B*) Percentage of total enzyme bound to beads.

tein distribution. NAGT I could not be visualized in the Triton pellet (lane *3*) because it is a less abundant protein than Mann II, as calculated from the purification tables published by Moremen et al. (1991) and Nishikawa et al. (1988). These data show that only *medial*-Golgi enzymes were selectively retained in the Triton pellet.

To determine whether Mann II and NAGT I were present in the same or different structures, magnetic beads were coated with either a control monoclonal antibody to human CD8 (OKT8) or one to Mann II (53FC3; Burke et al., 1982) and incubated with increasing amounts of the resuspended Triton pellet. To minimize non-specific binding it was necessary to remove TX-100 from the pellets and resuspend them in PBS containing BSA. Salt solubilizes the *medial*-enzymes (see below) but does so less readily in the absence of TX-100 and no more than 20% of the Mann II was removed from the pellet under these conditions (data not shown).

Both Mann II and NAGT I bound to the beads coated with anti-Mann II but not control antibodies (Fig. 3 *A*). The amount of Mann II bound increased with increasing amounts of added Triton pellet proteins and correlated closely with the binding of NAGT I. Furthermore, the percentage of Mann II bound decreased with increasing amounts of pellet proteins as the beads became saturated (Fig. 3 *B*). This decrease was closely matched by that for NAGT I, though the percentage bound was slightly lower, mimicking its slightly greater solubility during Triton extraction. These data show that NAGT I and Mann II are present in the same large structures in the Triton pellet.

Beads containing bound Mann II and NAGT I were prepared for electron microscopy. Fig. 4 *A* shows an untreated Golgi stack, and Fig. 4 *B* shows Golgi stacks after extraction with 2% TX-100. Intermediate concentrations of TX-100 showed that the dilated rims were the first to disappear followed by the fragmentation and partial unstacking of cisternal membranes (data not shown). Low levels of TX-100 do not remove all the lipid from membranes (Helenius and Simons, 1975) and, in many cases, do not affect the morphology of the depleted bilayer (for example see Cotman et al., 1971; Helenius and Söderlund, 1973). Both the width (100 Å) and tri-laminar appearance of the membranes were unaltered (Fig. 4, compare *A* with *B*) and they usually appeared to be partially stacked. The length of the fragments was 169 nm ± 103 (± SD, n = 102). There was, in addition, amorphous material which varied from preparation to preparation and region to region of the pellet (Fig. 4 *B*). This material often appeared to emanate from the ends of the membrane remnants suggesting it was derived from them. As shown in Fig. 4, *C–E*, both the membrane fragments and the amorphous material were bound to the beads. Careful examination showed that both could be found closely apposed to the bead surface, indicating that both contained Mann II.

Additional evidence that both enzymes were present in the same structure was obtained by incubating the resuspended Triton pellets with either control or anti-Mann II antibodies followed by anti-mouse antibodies coupled to 10 nm colloidal gold. After centrifugation to equilibrium both enzymes equilibrated at 68% (wt/vol) sucrose with control antibodies whereas both were found at the bottom of the tube in the presence of anti-Mann II antibodies (data not shown).

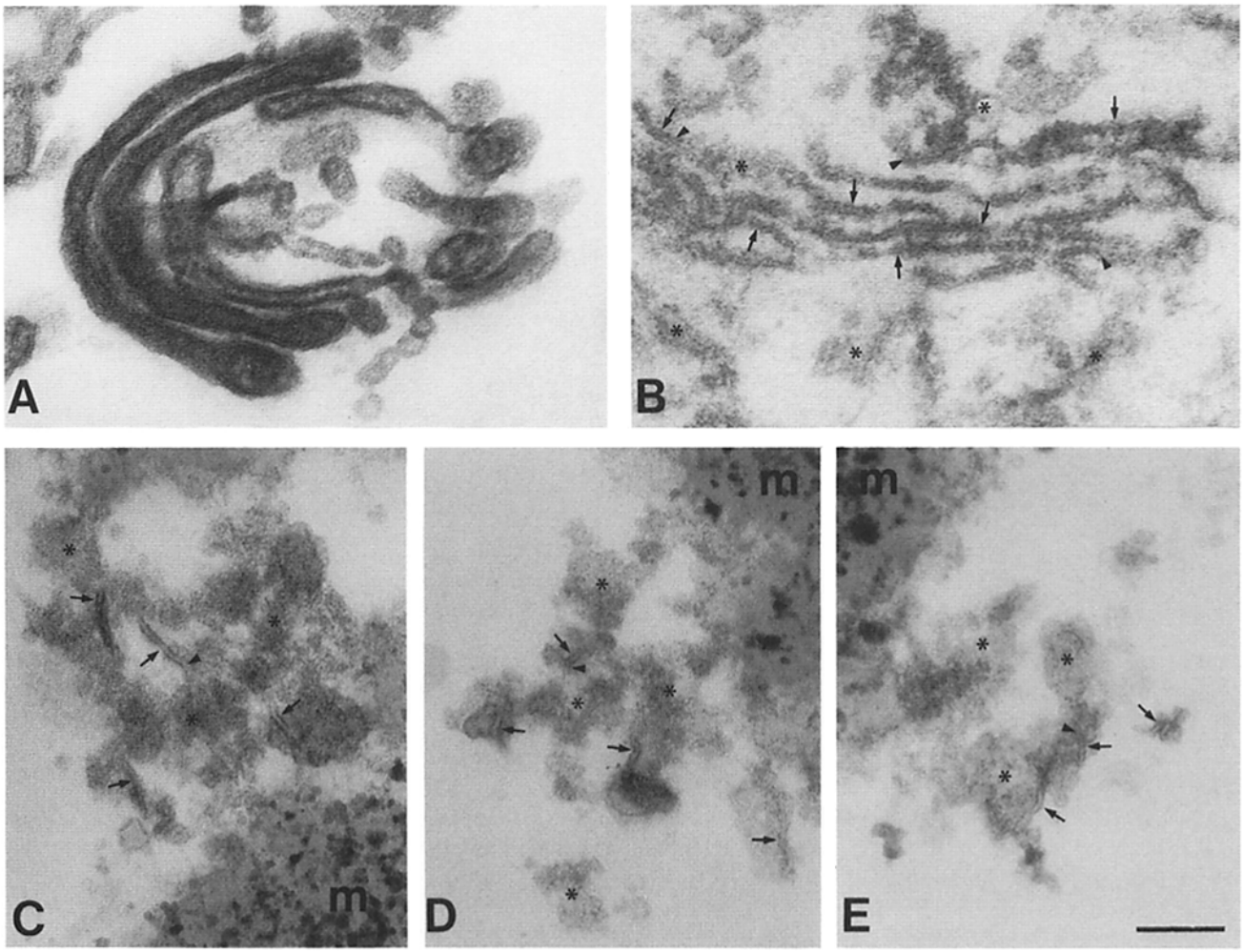

Figure 4. Electron microscopy of rat-liver Golgi stacks and Triton pellet fractions before and after immuno-isolation. Rat-liver Golgi stacks (*A*) were extracted with TX-100 and the pellets (*B*) incubated with magnetic beads (*m*) coated with anti-Mann II antibodies (53FC*3*), followed by several washing steps (*C–E*). All samples were fixed with glutaraldehyde and prepared for electron microscopy. Note the membrane remnants (*arrows*) with a tri-laminar appearance which are partially stacked in *B*. The amorphous material (*) was occasionally seen to emanate from the remnants (*arrowheads*). Bar, 100 nm.

Salt Solubilization and Reconstitution

Triton pellets were extracted with increasing concentrations of NaCl followed by centrifugation at low speed to yield a salt supernatant and pellet fraction. As shown in Fig. 5 *B*, both NAGT I and Mann II were readily solubilized, with complete solubilization occurring at ~120 mM, though this value did vary slightly from preparation to preparation. Recoveries of the enzyme were generally close to 100% (Fig. 5 *A*) showing that little, if any, of the enzymes remained bound to the salt pellet in an inactive state.

The solubilized enzymes both behaved as dimers on sucrose gradients containing 2% TX-100 (data not shown) and the released Mann II could be visualized by SDS-PAGE (Fig. 1, see lanes *4* and *5*). The salt pellet contained ~10% of the original Golgi proteins and contained 12 major proteins ranging in molecular weight from 14–200 kD (Fig. 1, lanes *6* and *7*). This pellet could also rebind both NAGT I and Mann II after dialysis to remove the salt (Fig. 6). Triton pellets were extracted with 150 mM salt to prepare salt pellets and with 70 mM salt to prepare supernatants containing the *medial*-enzymes but lacking any salt pellet proteins, since initial experiments showed that the pellet proteins were partially solubilized at 150 mM salt (data not shown). Approximately 75% of the Mann II and 60% of the NAGT I were extracted at this lower salt concentration (see Fig. 5 *B*). As shown in Fig. 6 the rebinding of both enzymes required both the salt pellet and dialysis.

Two lines of evidence showed that the rebinding was specific. Rat liver Golgi stacks were extracted simultaneously with 2% TX-100 and 150 mM salt to solubilize all Golgi markers and then centrifuged at low speed after dialysis. As shown in Fig. 7 both Mann II and NAGT I rebound after dialysis but not GalT.

The other line of evidence exploited the specific activity of purified Mann II (Moremen et al., 1991). This allowed the concentration of the enzyme in the salt supernatant and pellet to be calculated so that the apparent dissociation constant could be determined using Scatchard analysis. A fixed amount of salt pellet was incubated with varying amounts of low-salt supernatant containing solubilized Mann II. 25 mM salt was used for the preparation of these supernatants to en-

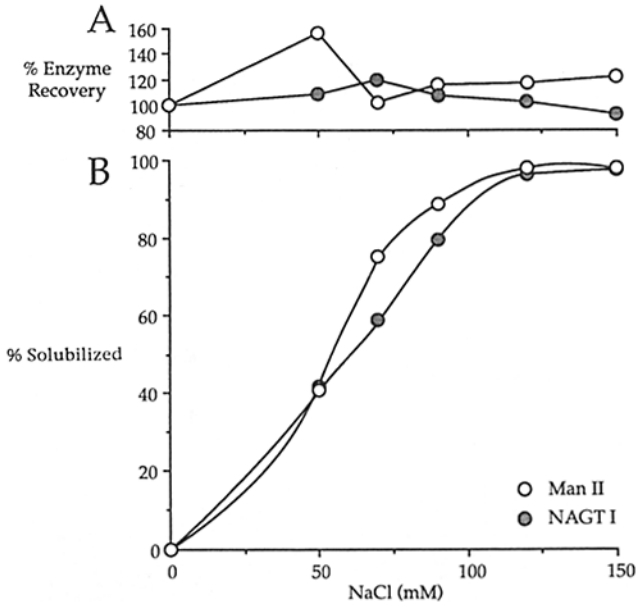

Figure 5. Extraction of the Triton pellet fraction with NaCl. Triton pellets were treated with increasing concentrations of NaCl, centrifuged at low speed and the levels of Mann II and NAGT I in the supernatant and pellet measured. (*A*) Percentage recovery of enzyme activity relative to untreated sample. (*B*) Percentage of total enzyme solubilized.

sure that no solubilization of the pellet proteins had occurred. Fig. 8 shows a representative Scatchard analysis from the four experiments performed, which yielded an average apparent dissociation constant of 3.0 nM ± 0.2

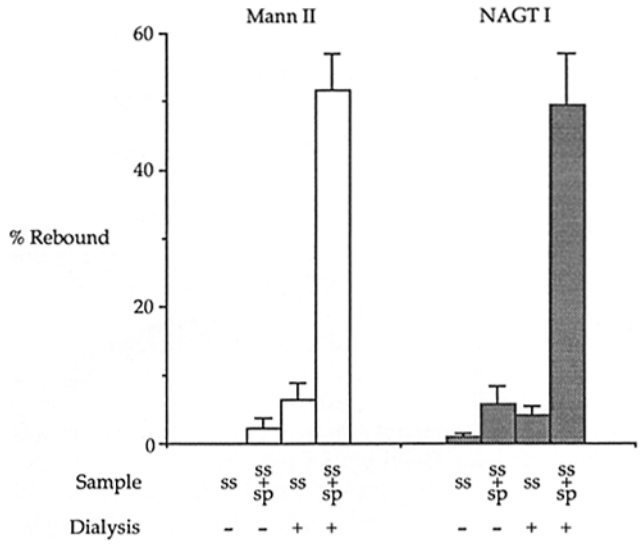

Figure 6. Reconstitution of binding to the salt pellet. Triton pellet (from 100 μg Golgi stacks) was extracted with 70 mM salt and the supernatant (*ss*) incubated alone or with a salt pellet (*sp*) obtained after extraction of a Triton pellet (from 100 μg Golgi stacks) with 150 mM salt. Samples were either dialyzed or incubated at 4°C. After centrifugation at low speed, the percentage of enzyme activities in the reconstituted pellet was calculated ±SEM (n = 3). The recovery of enzyme activity in the dialyzed samples compared with the undialyzed salt supernatant was 96% ± 11.3 (Mann II) and 92% ± 7.2 (NAGT I).

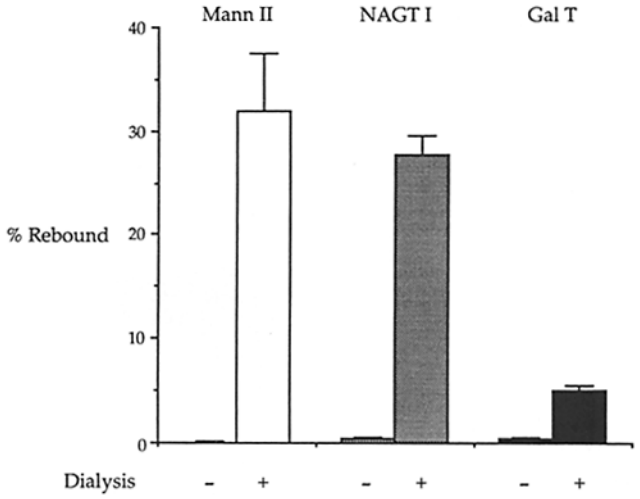

Figure 7. Specificity of re-binding after reconstitution. Rat liver Golgi stacks were extracted simultaneously with 2% TX-100 and 150 mM NaCl, and then either dialyzed or incubated at 4°C followed by centrifugation at low speed. The percentage of enzyme activity in the reconstituted pellet was calculated ±SEM (n = 3). The recovery of enzyme activity in the dialyzed samples compared with the undialyzed was 108% ± 2.4 (Mann II), 119% ± 3.6 (NAGT I), and 106% ± 1.6 (GalT).

(±SEM, n = 4) for the Mann II dimer. Such a low value indicates that Mann II binds with very high affinity to the salt pellet and argues strongly that binding is specific. Scatchard analysis was also performed for NAGT I and yielded an apparent dissociation constant of 70pM ± 20 (±SEM, n = 4). This value should be treated with a certain amount of caution since it was calculated using a specific activity derived for the rabbit, not rat, enzyme and the substrate used was the purified oligosaccharide, not the intact glycoprotein (Oppenheimer and Hill, 1981). However, this does demonstrate that NAGT I is also tightly bound to the matrix, with an affinity in the same range as that for Mann II.

Treatment with Proteases

The salt pellet contains a large matrix which specifically binds to *medial*-Golgi enzymes. This matrix could be within the lumen of *medial*-cisternae or in the intercisternal spaces. Its topology was determined using proteinase K.

Golgi stacks were incubated with or without 0.5 mg/ml proteinase K for 30 min at 25°C. After quenching with PMSF, Triton pellets and supernatants were prepared and the percentage of *medial*-enzymes in the pellet determined. As shown in Fig. 9 *A* high levels of Mann II and NAGT I were found in the Triton pellets from mock-treated Golgi stacks but much lower amounts from those digested with proteinase K. The protease did not digest the enzymes themselves because recoveries from digested samples were close to 100% compared with mock-treated controls. Parallel electron microscopy showed that the Golgi membranes were unaffected by mock treatment but were unstacked by digestion (data not shown). The increase in solubility of these enzymes after digestion and TX-100 extraction could be explained in two ways. First, the protease had digested a cytoplasmic matrix to which the *medial*-enzymes were bound or, second,

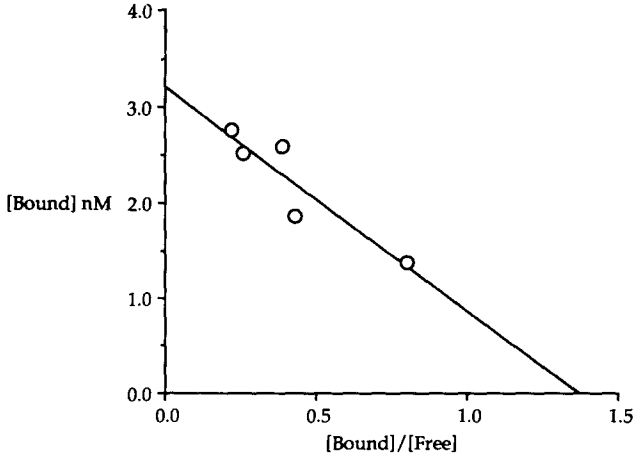

Figure 8. Scatchard analysis of the rebinding of Mann II. A 150-mM salt pellet was incubated with increasing amounts of 25 mM salt supernatant, dialyzed, and sedimented at low speed. The concentration of bound and free Mann II dimers was calculated from the enzyme activity and the known specific activity of the purified protein. A representative graph from the four experiments carried out is shown and the average recovery of enzyme activity after dialysis was 100% ± 4.2 (±SEM).

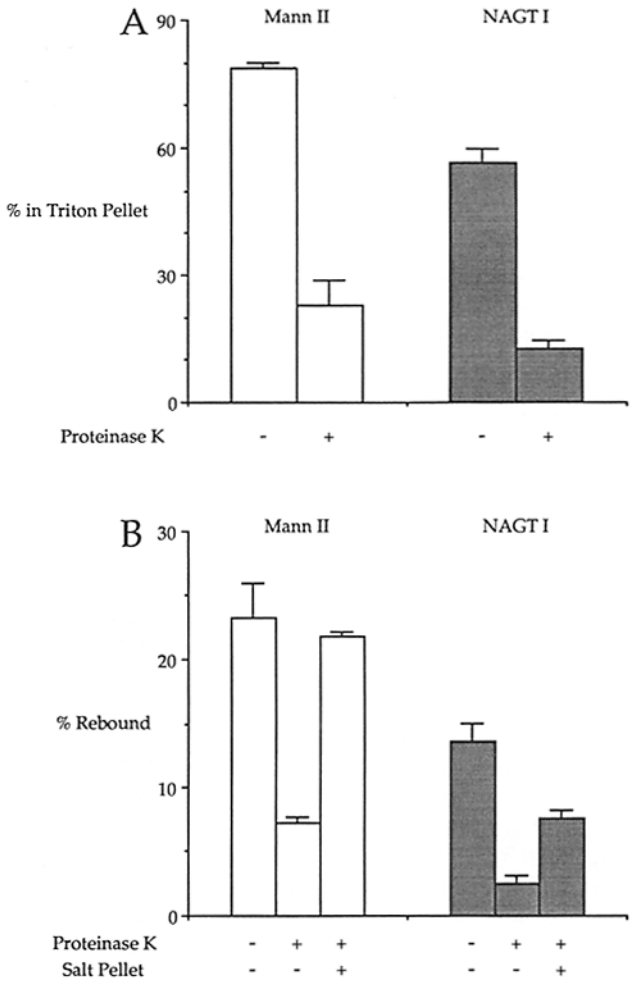

Figure. 9. Protease treatment of intact Golgi stacks. (*A*) Rat liver Golgi stacks were incubated in the presence or absence of proteinase K. After quenching with PMSF, Triton pellets were prepared and the percentage of bound Mann II and NAGT I calculated ±SEM (n = 3). The recovery of Mann II compared with untreated Golgi stacks was 76% ± 2.5 for the mock-treated and 79% ± 1.0 for the digested sample, and for NAGT I was 75% ± 0.9 for the mock-treated and 66% ± 3.2 for the digested sample. (*B*) Rat liver Golgi stacks were mock-treated or treated with proteinase K and then incubated simultaneously with both 2% TX-100 and 150 mM salt to solubilize the Golgi enzymes. A 150 mM salt pellet (from untreated Golgi stacks) was added to one of the treated samples before dialysis and low speed centrifugation. The percentage of Mann II and NAGT I in the pellet was calculated ±SEM (n = 3). The recovery of enzyme activity in the digested samples compared to the mock was 106% ± 3.6 (Mann II) and 103% ± 3.1 (NAGT I).

that a conformational change induced by digestion of the enzymes' cytoplasmic tails abolished their binding to a lumenal matrix. To distinguish between these possibilities, Golgi stacks were incubated with or without proteinase K followed by simultaneous extraction with 2% TX-100 and 150 mM salt to completely solubilize both enzymes. After dialysis the mock-treated pellet contained both of the enzymes whereas the treated pellet did not. Addition of a salt pellet from untreated Golgi stacks to the digested samples, however, restored the sedimentability and therefore demonstrated that the enzymes were intact (Fig. 9 *B*). The level of Mann II rebinding on addition of the salt pellet was 94% that of mock-treated Golgi stacks whereas the level of rebound NAGT I was 56%.

Discussion

By extracting highly purified Golgi stacks with 2% TX-100 followed by 150 mM salt, we have isolated an insoluble matrix, that binds both NAGT I and Mann II with high affinity. Less than 20% of the other Golgi enzymes and proteins, all from other parts of the Golgi apparatus, were bound. The only exception was the CGN marker, p58, 35% of which was present in the Triton pellet. This, however, contrasts with 77% of the NAGT I and 86% of the Mann II. These high percentages, coupled with the presence of both *medial*-enzymes throughout the cisternae in which they reside (Burke et al., 1982; Nilsson et al., 1993*a*), show that the matrix must be an extensive structure. Its sensitivity to proteinase K also suggests that it is in the intercisternal space.

The large size and position of this structure are borne out by its rapid sedimentation and morphological appearance. Short stretches of what appear to be cisternal membrane remnants were found after TX-100 extraction (Fig. 4 *B*) and after binding to beads coated with anti-Mann II antibodies (Fig. 4, *C–E*), though these bound remnants were often much shorter. They had the same width (100 Å) as the cisternal membranes in the original Golgi stacks (Fig. 4 *A*) and the same tri-laminar appearance.

In addition to the cisternal remnants there was also material which had an amorphous appearance. This material also contained Mann II since it was bound to the surface of beads coated with anti-Mann II antibodies (Fig. 4, *C–E*). On occasion it appeared to be continuous with the ends of the cisternal remnants (Fig. 4, *B–E*), so the simplest interpretation is that it is an unravelled form of the matrix–enzyme complex. Normally the matrix would be tightly apposed to the cisternal membrane, bound to the *medial*-enzymes, but after partial removal of lipid by TX-100, the matrix could become un-

stable and unravel, thereby losing its tri-laminar appearance. Slight variations in the extent of lipid extraction could explain the variability in the amount of amorphous material from preparation to preparation and from one region of the pellet to another, and suggests that it is the lipid bilayer that gives the matrix its long range order. It also explains why cisternal remnants disappear after treatment with salt to remove *medial*-enzymes and do not re-appear after reconstitution (data not shown). Instead, amorphous material is observed, similar to that seen in Fig. 4, *B–E*. A flattened matrix is clearly not essential for binding since the efficiency after reconstitution was typically in excess of 50%.

The fact that most of the NAGT I and Mann II were present in the pellet after extraction with TX-100 also suggests that a binding site for each individual *medial*-enzyme exists, especially since purified Golgi enzymes such as Mann II exist as dimers in TX-100 (Tulsiani et al., 1977; Moremen et al., 1991) and do not interact with one another directly. The linearity of the Scatchard plot implies that only a single class of binding site exists for Mann II, and the apparent dissociation constant of 3 nM demonstrates tight binding to the matrix. Mann II could interact with the matrix either directly via the cytoplasmic tail, or indirectly, via the lumenal domain, to a membrane-spanning protein which itself binds to the matrix by its cytoplasmic domain. More work will be required to distinguish between these possibilities.

12 major proteins were found in the salt pellet. Some may be contaminants present in the original Golgi preparation while others might arise from matrices from different parts of the Golgi stack that are responsible for binding *cis*- and *trans*-Golgi enzymes. They may simply not function under the re-binding conditions used. Some might be contained within those structures observed by electron microscopy. The proteins responsible for binding Mann II and NAGT I will only be identified after successful solubilization and reconstitution of the binding activity in the salt pellet. Preliminary work suggests that solubilization with 8 M urea is a promising approach and fractionation of urea extracts should lead to purification of the binding proteins.

The function of *medial*-enzyme binding to the matrix is unclear. Retention of Golgi enzymes and proteins in the stack is mediated by the membrane spanning domain, not the cytoplasmic or lumenal domains (Machamer, 1993; Nilsson et al., 1993*b*), so binding to the matrix could, at best, only enhance the retention process. A role in stacking is a possibility since proteolysis of the matrix unstacks the cisternae. Furthermore, NAGT I has been localized to both the *medial*- and *trans*-cisternae (Nilsson et al., 1993*a*) so stacking could result if the NAGT I molecules in these cisternae bound to the same matrix. Identification of the enzyme binding protein(s) might, therefore, give insight into mechanism that stacks the cisternae.

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