In Vitro Synthesis of Sulfated Glycosaminoglycans Coupled to Inter-compartmental Golgi Transport*

Carlos J. Fernández‡ and Graham Warren§
From the Imperial Cancer Research Fund, Cell Biology Laboratory, 44 Lincoln’s Inn Fields, London WC2A 3PX, United Kingdom

We have used isolated rat liver Golgi membranes to reconstitute the synthesis of sulfated glycosaminoglycans (GAGs) onto the membrane-permeable, external acceptor xyloside. Biosynthetic labeling of GAGs with $[^{35}S]$sulfate in vitro is shown to have an absolute requirement for ATP and cytosolic proteins and is inhibited by dismantling the Golgi apparatus with okadaic acid or under mitotic conditions suggesting that inter-compartmental transport between Golgi cisternae is a prerequisite for the successful completion of the initiation, polymerization, and sulfation of GAGs. Accordingly, we show that in vitro synthesis of $^{35}S$-GAGs utilizes the same machinery employed in Golgi transport events in terms of vesicle budding (ADP-ribosylation factor and coatomer), docking (Rabs), targeting (SNAREs), and fusion (N-ethylmaleimide-sensitive factor). This provides compelling evidence that GAGs synthesis is linked to Golgi membrane traffic and suggests that it can be used as a complementation-independent method to study membrane transport in Golgi preparations from any source. We have applied this system to show that intra-Golgi traffic requires the function of the Golgi target-SNARE, syntaxin 5.

Glycosaminoglycans (GAGs)¹ are sulfated polysaccharides that share a common pattern of structure consisting of an unbranched, alternating sequence of hexosamine (GlcN, GlcNAc, or GalNAc) and hexuronic acid (GlcUA or IdcEA).

The biosynthesis of GAG chains involves the ordered, stepwise addition of a series of carbohydrate units onto the polypeptide backbone of a proteoglycan (for reviews see Refs. 1–3). The process starts with the addition of xylose (Xyl) to a Ser or Thr residue of the protein backbone. Two Gal residues and a GlcUA unit are then added sequentially to form the tetrasaccharide core linkage region (GlcUA-Gal-Gal-Xyl) common to most GAGs. Chain polymerization then starts with the addition of GlcNAc (in heparin/heparan sulfate) or GalNAc (in chondroitin sulfate and dermatan sulfate) residues alternating with GlcUA to form a chain containing up to 100 or more monosaccharide units. Polymerized sugars are then subjected to a series of modifications including N-deacetylation/N-sulfation, O-sulfation, and epimerization of GlcUA to IdcEA.

It is important to note that many of these substitutions do not occur at random but only take place in a coordinated fashion after a previous modification has rendered the appropriate substrate available to the next modifying enzyme(s). Thus, the enzyme adding the second Gal in the linkage region (Gal transferase II) will only do so after the product of Gal transferase I (Xyl-Gal) is formed. N-Deacetylation (in heparan sulfate) is necessary for N-sulfation to occur, and N-sulfation is an absolute prerequisite for C5 epimerization of GlcUA to IdcEA and for O-sulfation (4). N-Sulfation of the entire chain is terminated before O-sulfation is initiated (5). The biosynthetic process is fast; complete synthesis of an entire GAG chain is achieved in 1–3 min (6).

All the transporters necessary to translocate the sugar precursors (UDP-monosaccharide) and the sulfate donor (adenosine-3'-phospho-5'-phosphosulfate, PAPS) used as building blocks for GAGs are located on Golgi membranes (7). With the possible exception of Xyl addition to the polypeptide backbone, which probably occurs in the endoplasmic reticulum (ER), the biochemical machinery for the synthesis of the tetrasaccharide linkage region and for the chain polymerization, epimerization, and sulfation of GAGs is located inside the lumen of the Golgi apparatus (2, 8, 9).

Several lines of evidence suggest that the biochemical reactions for the synthesis of sulfated GAGs take place in separated membrane compartments of the Golgi complex and that, therefore, the GAG precursors attached to the backbone protein must be transported from one cisterna to the next one along the secretory pathway to undergo the ensuing biosynthetic reactions. Thus the first Gal addition by Gal transferase I to Xyl-Ser is a very early, probably cis-Gal-G, modification, whereas addition of the second core Gal (by Gal transferase II) and GlcUA occur in a more distal compartment (at least medial-Golgi), as do the polymerization reactions, whereas sulfation is primarily a trans-Golgi modification. Evidence for this model comes from experiments in which intracellular membrane traffic is stopped either by the absence of cytosol or by the inclusion of transport inhibitors such as N-ethylmaleimide (NEM) or ilimaquinone; under these conditions GAG synthesis does not take place and only Gal-Xyl is made, with very little production of Gal-Gal-Xyl (10). This suggests that Gal transferases I and II are largely located in separate membrane compartments, a conclusion in good agreement with subfractionation studies in which both enzymes were shown to have a different density distribution (11). Another standard procedure to inhibit intracellular transport, treatment with brefeldin A (BFA), has been shown to block GAG synthesis in many systems (12–17). Even
in vivo process in terms of transport conditions, fusion, and coat proteins required. Additionally we show that the Golgi t-SNARE syntaxin 5 is involved in this process.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Chondroitinase AC was purchased from Oxford Glyco-systems; staurosporine and okadaic acid were from Calbiochem; [35S]PAPS (2.5 Ci/mmol) and Na2[35SO4] (carrier-free) were from NEN Life Science Products; and GTP-s were from Boehringer Mannheim. All other chemicals were from Sigma.

**In Vitro Golgi Transport of Glycosaminoglycans**

**Cell Culture and Labeling with [35S]Sulfate**—As virtually all cells tested are capable of synthesizing GAGs onto xyloside, an assay in which xyloside-attached GAGs, would provide a simple test to assess transport ability, should have universal application, and could be utilized to standardize Golgi transport characteristics and requirements across a wide range of systems. We decided, therefore, to attempt to establish such a system using isolated rat liver Golgi and xyloside.

We describe here the development of such an assay and show that sulfation of newly synthesized GAGs onto externally added xyloside is indeed coupled to intra-Golgi transport. Our evidence demonstrates that this system closely follows the characteristics of the in vivo process in terms of transport conditions, fusion, and coat proteins required. Additionally we show that the Golgi t-SNARE syntaxin 5 is involved in this process.

**EXPERIMENTAL PROCEDURES**

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**Cell Culture and Labeling with [35S]Sulfate**—Mitotic HeLa cells obtained as above were used for the estimation of the [35S]-GAGs synthesis size. After the 24 h nocodazole treatment (resulting in ≥95% mitotic cells), 5 × 106 cells were allowed to attach for 2 h to 6-well plastic dishes coated with polylysine. The medium was then removed, and the cells were washed three times with PBS and incubated in buffer containing 5 mM NaCl, 5.4 mM KCl, 0.9 mM Na2HPO4, 10 mM MgCl2, 2 mM CaCl2, 1 g/liter glucose, 0.1 g/liter Hepes, pH 7.4, containing 0.1 mM xyloside as well as 0.1 μg/ml nocodazole for 20 min at 37 °C after which the medium was removed and 0.5 ml of [35S]-sulfate (0.25 μCi/ml) in buffer A was added. After 10 min of labeling at 37 °C the medium was removed, and the cells were washed five times with 20 mM unlabeled sulfate in PBS. Cells were then extracted and processed for [35S]-GAGs determination by the cetylpyridinium chloride (CPC) precipitation technique as described (27).

For okadaceous treatment, 4 × 105 HeLa cells plated in 6-well plastic dishes were incubated with xyloside as above and then supplemented with 5 μM okadaceous from a 2 mM stock solution made in 10% Me2SO and incubated for 30 min at 37 °C after which they were labeled for 5 min with 0.125 μCi of [35S]-sulfate. Cells were then extracted for [35S]-GAGs measurement as above.

For the experiment described in Fig. 3C, mitotic HeLa cells were obtained by selective detachment from roller bottles after synchronization with a double thymidine block as described (30). After the second treatment with thymidine the medium was washed, and dead cells were removed by high speed rolling. The cells were then allowed to grow for 3 h and treated with nocodazole. Such nocodazole-treated mitotic cells were then collected by rolling the culture bottles at 200 rpm. Nocodazole was then removed by washing three times in Eagle’s medium supplemented with 10% fetal calf serum, and cells were allowed to proceed out of mitosis in that medium in a spinner bottle. At different time points cell aliquots (0.5 ml, 2.5 × 106 cells) were taken out and incubated with xyloside (0.5 μg/ml and [35S]-sulfate (50 μCi) for 10 min at 37 °C. Cells were then chilled on ice, washed three times with ice-cold PBS, and processed for determination of [35S]-GAGs by the CPC precipitation technique (27).

**Standard in Vitro Assay for 35S-GAGs Synthesis**—A typical reaction contained, in 50 μl, 10 μg of rat liver Golgi, 0.1 mg (protein) of HeLa cell interphase cytosol, 0.2 μm sucrose, 25 μM Hepes, pH 7.0, 25 mM KCl, 2.5 mM MgCl2, 1 mM MnCl2, 1 mM DTT, a mixture of UDP-sugars containing 0.5 μM each of UDP-Gal, UDP-GlcNAc, and UDP-GlcUA, an ATP regeneration system consisting of 1 mM ATP, 5 mM creatine phosphate, and 7 units of creatine kinase, 0.5 mM xyloside, and 0.5 μCi of [35S]-PAPS. This mixture was incubated for 30 min at 37 °C in borosilicate glass tubes. The reaction was stopped by addition of 100 μM of 7% trichloroacetic acid (final concentration). After 30 min on ice the samples were transferred to 1.5-ml microcentrifuge tubes and spun at 27,000 × g for 20 min. The supernatant was recovered and added to microcentrifuge tubes containing 1 ml of 0.25 M sodium acetate in methanol and kept at −20 °C or less for at least 1 h. After centrifugation at 27,000 × g for 30 min, the supernatant was discarded and the pellet air-dried and resuspended in 20 μl of electrophoresis sample buffer. 15 μl were then loaded onto 15% SDS-PAGE minigels and run at 120 V for 1 h. The gels were then briefly stained and de-stained and dried on paper. Dry gels were exposed in a PhosphorImager cassette for at least 1 day after which the accumulated radioactivity was quantified with the aid of a PhosphorImager scanner. We used this procedure for determination of [35S]-GAGs in our assay because the CPC precipitation technique (which measures the radioactivity present in the precipitate by scintillation counting) produced a very low signal in the in vitro assay despite working very well with living cells. Xyloside was omitted in some samples to measure background labeling. In some samples for the experiment depicted in Fig. 1A Na2[35SO4] (5 μCi) was used, instead of [35S]-PAPS. In some experiments mitotic, rather than interphase, cytosol was used (at a final concentration of 8 mg/ml), and in these cases the control reactions containing interphase cytosols received 1.5 mM EGTA and 2 mM β-glycerophosphate (present in the mitotic cytosols from the desalting step). This addition did not modify, by itself, the level of [35S]-GAGs synthesis.

For protease and glycogen digestion, the pellet from the methanol precipitation was dissolved in water and supplemented with the enzyme and a 100-fold concentrated aliquot of the appropriate buffer (according to the manufacturer) and incubated 24 h at 37 °C. Afterward the complexes received electrophoresis buffer and were run and processed as above.

Samples in which the effect of ATP was being verified received an ATP-depleting mixture consisting of (final) 10 mM glucose and 50 IU/ml hexokinase. The ATP-regeneration system was omitted in this case.

NEM-treated cytosol was prepared by adding 1 mM freshly prepared NEM (in water) to HeLa cell interphase cytosol for 15 min on ice and
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**RESULTS**

In Vitro Synthesis of GAGs by Rat Liver Golgi—Incubation of purified rat liver Golgi with xyloside, [35S]PAPS, and UDP-sugars under conditions that support vesicular transport (presence of cytosol and an ATP-regenerating system) resulted in the appearance of several bands corresponding to sulfated proteins as well as 35S-labeled material that ran as a smear in the gel. The C-terminal transmembrane domain (syntaxin 5–TM) was thereby referred to as soluble syntaxin 5. A His_{6}-tagged version of mutant Sar1 (H79G, GTP-restricted) was obtained as previously reported (37). His_{6}-tagged Rab-GDI was prepared according to Ullrich et al. (38).

Purification of p97 and Coatomer—Rat p97 was purified by anion exchange chromatography and density centrifugation as described (32). Coatomer was purified from rat liver cytosol as described by Waters et al. (39).

![Image](331x531)

**FIG. 1.** In vitro synthesis of xyloside-attached 35S-labeled GAGs. A, standard 50-μl reaction mixtures were incubated for 30 min at 37 °C in the presence of 0.5 μCi of [35S]PAPS (lanes 1 and 2) or 10 μCi of Na_2^{35}SO_4 (lanes 3 and 4). Xyloside (0.5 mM) was included in lanes 1 and 3. At the end of the incubation the mixture was treated with trichloroacetic acid and sodium acetate in methanol as described under "Experimental Procedures." The methanol precipitate was resuspended in sample buffer and run on a 15% SDS-polyacrylamide gel, and the labeled material was analyzed as described under "Experimental Procedures." Molecular mass standards (in kDa) appear on the left. B, quantification of radioactive material along lanes 1 (+ xyloside, ●) and 2 (– xyloside, ○). Each point stands for the radioactivity measured in cells from a grid composed of 20 cells of 10 × 3 mm superimposed on the image of the scanned lane using the program NIH Image. The abscissa (R_f) depicts the distance of the scanned point relative to the front of the gel.

then quenching with 3 mM DTT on ice for 5 min. The controls received in this case a pre-quenched mixture of 1 mM NEM and 3 mM DTT.

Recombinant Proteins—Bacterially expressed, His_{6}-tagged NSF and α-SNAP were purified by nickel affinity chromatography as described previously (31, 32). Recombinant myristoylated ARF1 (33) and p47 (34) were prepared as described. For the preparation of recombinant, soluble syntaxin 5, the cDNA coding for the p35 form (see Ref. 35) minus the C-terminal transmembrane domain (syntaxin 5–TM) was amplified by polymerase chain reaction and the fragment obtained cloned into the pGEX4T-2 vector. GST-syntaxin 5–TM was thereby purified on glutathione-Sepharose as described (36). Syntaxin 5–TM is hereby referred to as soluble syntaxin 5. A His_{6}-tagged version of mutant Sar1 (H79G, GTP-restricted) was obtained as previously reported (37). His_{6}-tagged Rab-GDI was prepared according to Ullrich et al. (38).

Coatomer was purified from rat liver cytosol as described by Waters et al. (38). (39).

![Image](607x83)

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In order to define further the nature of the 35S-GAGs obtained from the in vitro incubation, we subjected them to hy-
Digestion of low molecular weight $^{35}$S-labeled products obtained from a typical in vitro reaction. 20 μg of rat liver Golgi membranes were incubated with 0.2 ng (protein) of HeLa cell cytosol for 30 min at 37 °C in the presence of HKM buffer, 0.2% sucrose, an ATP-regenerating system, 0.5 mM xyloside, a mixture of UDP-sugars containing 0.5 mM each of UDP-Gal, UDP-GlcNAC, and UDP-GlcUA, as well as 2 μCi of $[^{35}]$PAPS, all in 100 μl. At the end of the incubation 7% trichloroacetic acid (final concentration) was added on ice and the sample spun at 27,000 × g for 30 min. The supernatant was recovered and added to 1 ml of 0.25 M sodium acetate in methanol as above, and the pellet thus obtained was resuspended in 20 μl of sample buffer and loaded onto 15% SDS-polyacrylamide gels. After running, the gels were briefly stained and de-stained, and then dried and exposed in a PhosphorImager cassette. $^{35}$S-Labeled products were quantified with a PhosphorImager device. Values are given as a percent of the control (no digestion) and represent the mean of two experiments.

| Enzyme                        | $^{35}$S-GAGs remaining (% control) |
|-------------------------------|-----------------------------------|
| No addition                  | 100                               |
| Heparitinase-I                | 28                                |
| Chondroitinase AC             | 77                                |
| Chondroitinase ABC            | 79                                |
| Heparitinase-I + chondroitinase ABC | 7                   |
| Pronase E                     | 103                               |
| Proteinase K                  | 98                                |

In vitro synthesized $^{35}$S-GAGs were found inside the Golgi membranes as they were retained in the pellet after centrifuging the reaction mixture at 27,000 × g for 15 min but remained in the supernatant if membranes were treated with 0.05% Triton X-100 after the biosynthetic reaction (data not shown).

In vitro synthesis of $^{35}$S-GAGs was linked to membrane (presumably vesicular) transport. We first ruled out the possibility of synthesis by glycosylation of material that had diffused out of the lumen. Thus, membrane permeabilization with 0.05% Triton X-100 during the reaction abolished synthesis of sulfated GAGs (Fig. 2A) suggesting that properly sealed vesicles and cisternae are needed for efficient synthesis as would be expected from a process driven by vesicular transport.

Synthesis showed an absolute requirement for ATP and cytosolic proteins (Fig. 2A). It did not take place at 4 °C. Synthesis continued approximately linearly up to about 30 min of incubation (Fig. 2B). Increasing cytosol concentration up to about 2 mg/ml linearly increased the amount of sulfated GAGs obtained (not shown).

If polymerization and sulfation of GAGs are coupled to membrane transport, it could be predicted that procedures that interfere with membrane traffic should also affect GAGs synthesis. Therefore, we decided to measure, in HeLa cells, the extent of xyloside-induced synthesis of sulfated GAGs after treatment with okadaic acid, a phosphatase inhibitor known to arrest membrane traffic and fragment the Golgi apparatus (49). Fig. 3A shows that preincubation with 5 μM okadaic acid completely abolished the in vitro synthesis of $^{35}$S-GAGs onto xyloside.

Another well established condition under which membrane traffic is inactivated is mitosis. Entry into mitosis brings about the dismantling of the Golgi apparatus (50) and a general cessation of membrane transport including intra-Golgi transport (29, 51–53). HeLa cells arrested in metaphase by a 24-h treatment with 0.1 μg/ml nocodazole show a 90% decrease in their capacity to synthesize $^{35}$S-GAGs onto xyloside compared to their capacity for synthesis in interphase.
were incubated with 0.5 mM xyloside for less than 5%). The one obtained in its absence (usually activity in the presence of xyloside minus the one obtained in its absence (usually less than 5%)).

The values shown are the mean ± S.E. of triplicate samples representing net synthesis of xyloside-attached35S-GAGs (i.e. radioactivity in the presence of xyloside minus the one obtained in its absence (usually less than 5%)).

B, spinner HeLa cells were arrested in mitosis by 24 h treatment with nocodazole and then allowed to attach to plastic dishes for 2 h. Xyloside (0.5 mM) was then added for 30 min after which the cells were labeled for 10 min with [35S]sulfate. Immediately afterward cells were extracted and processed as above. The values of net synthesis of xyloside-attached35S-GAGs are given as cpm/μg of protein to take into account a higher number of mitotic cells detaching from the dishes during the procedure. The means ± S.E. of triplicate samples are shown.

C, HeLa cells were synchronized by a double-thymidine block, collected by shake-off, and then arrested in pro-metaphase by a 4-h treatment with nocodazole after which this drug was washed off and the cells allowed to proceed out of mitosis. At the indicated times after this, cell aliquots were taken and the mitotic index (percent of cells remaining in mitosis) measured. A parallel cell aliquot was incubated for 10 min with xyloside and [35S]sulfate after which the newly synthesized35S-GAGs were quantified as described above.

It is thus evident that arrest of membrane transport will block synthesis of GAGs in living cells. We wanted to test whether this was also the case in our in vitro assay. Addition of 5 μM okadaic acid to the reaction mixture decreased35S-GAGs by 50% (Fig. 4A), and this effect was counteracted by the protein kinase inhibitor staurosporine which alone nearly doubled the control signal indicating that the extent of membrane traffic in vitro can be modulated by protein phosphorylation in a manner resembling the situation in vivo (56, 57). Substituting mitotic cytosol from HeLa cells for the standard interphase cytosol brings about a 60–70% inhibition of35S-GAG synthesis (Fig. 4B). This compares with about 50% inhibition of intragolgi transport by mitotic cytosol obtained using the transport assay developed by Rothman and co-workers (29). A 20-min preincubation with mitotic cytosol (Fig. 4B) gave essentially the same results indicating that the mitotic effect is fast. In our hands mitotic cytosol-induced block of GAG synthesis requires high concentration of cytosol (above 7 mg protein/ml) and a cytosol stabilized in the mitotic state by addition of EGTA and β-glycerophosphate. It is interesting to note that these were the conditions necessary to achieve mitotic disassembly of Golgi membranes in vivo (58) indicating, as has already been suggested (59, 60), that both processes are related.

NSF, Rabs, and COPI but Not COP-II Components Are Used in GAGs Transport—The N-ethylmaleimide-sensitive factor

FIG. 3. Dismantling of the Golgi apparatus during mitosis or by okadaic acid treatment inhibits35S-GAGs synthesis in vivo. A, cultured HeLa cells were incubated with 0.5 mM xyloside for 20 min and then okadaic acid (5 μM) was added for 30 min after which the cells were pulse-labeled with [35S]sulfate for 5 min. Cells were then extracted, and the amount of35S-GAGs was determined by the CPC precipitation technique (27). The values shown are the mean ± S.E. of triplicate samples representing net synthesis of xyloside-attached35S-GAGs (i.e. radioactivity in the presence of xyloside minus the one obtained in its absence (usually less than 5%). B, spinner HeLa cells were arrested in mitosis by 24 h treatment with nocodazole and then allowed to attach to plastic dishes for 2 h. Xyloside (0.5 mM) was then added for 30 min after which the cells were labeled for 10 min with [35S]sulfate. Immediately afterward cells were extracted and processed as above. The values of net synthesis of xyloside-attached35S-GAGs are given as cpm/μg of protein to take into account a higher number of mitotic cells detaching from the dishes during the procedure. The means ± S.E. of triplicate samples are shown.

C, HeLa cells were synchronized by a double-thymidine block, collected by shake-off, and then arrested in pro-metaphase by a 4-h treatment with nocodazole after which this drug was washed off and the cells allowed to proceed out of mitosis. At the indicated times after this, cell aliquots were taken and the mitotic index (percent of cells remaining in mitosis) measured. A parallel cell aliquot was incubated for 10 min with xyloside and [35S]sulfate after which the newly synthesized35S-GAGs were quantified as described above.

with interphase cells (Fig. 3B). This result agrees with an early study with CHO cells (54) in which a 3–4-fold inhibition of GAGs synthesis under mitosis was reported. The decrease in GAG synthesis could also be interpreted as the consequence of the effect of nocodazole per se on the GAG biosynthetic machinery or the result of widespread cell death after 24 h with the drug rather than a specific effect arising from the mitotic condition. The first possibility is rendered less likely by the observation that short-term treatment of interphase cells with 0.1 μg/ml nocodazole had no effect on GAG synthesis (not shown). To address the second possibility mitotic HeLa cells were obtained by shake-off after synchronization with a double thymidine block followed by a 4-h treatment with nocodazole and then allowed to enter interphase after washing away the nocodazole. Subsequently, aliquots of cells were taken at different time points and incubated with xyloside and [35S]sulfate to induce synthesis of labeled GAGs. Fig. 3C shows the results of this experiment. It is clear that the mitotic block is reversible and that xyloside-induced synthesis of 35S-GAGs mirrors the fall in the mitotic index (percent of total population of cells undergoing mitosis). Just 1 h after nocodazole removal the cells recovered their full GAG synthesizing capacity. At this stage, when most cells were entering early G1, we saw an overshoot of GAGs synthesis very similar to the one reported in the autoradiographic study of Preston et al. (55).
(NSF) is a protein first isolated as an NEM-inactivated component required at the fusion stage of intra-Golgi transport (61, 62) and later shown to be crucial, by biochemical and genetic criteria, for many steps of inter-compartmental transport (63, 64). To address the involvement of this factor in our \textit{in vitro} GAG assay, the reaction mixture (both membranes and cytosol) was treated with 1 mM NEM on ice for 15 min, after which the remaining reagent was quenched by addition of 3 mM DTT. This manipulation inhibited xyloside-induced \textsuperscript{35}S-GAG synthesis. However, the effect could not be reversed by addition of fresh cytosol or purified recombinant NSF (data not shown). The irreversibly inactivated process lies in the Golgi membranes and not in the cytosol. It is likely that this is the N-deacetylation of heparan sulfate, a modification sensitive to 1 mM NEM (65) and an activity absolutely required for subsequent sulfation of the GAG chain (4). Treatment of cytosol alone with 1 mM NEM inactivated by 60–70\% the capacity of isolated Golgi membranes to synthesize \textsuperscript{35}S-GAGs (Fig. 5). The activity was restored by addition of 0.5 \mu g of purified recombinant NSF (added together with 1.5 \mu g of \alpha-SNAP). Addition of NSF plus \alpha-SNAP to intact cytosol had no effect (not shown) suggesting that normal levels of cytosolic NSF are near-saturating. Only a slight recovery could be achieved by addition of purified rat p97 (Fig. 5), another NEM-sensitive protein believed to be involved in some homotypic, rather than heterotypic, membrane fusion events (32, 66, 67). Our purified p97 was depleted in p47, a protein believed to be an obligatory partner in p97 function (34). Nevertheless, even when we supplemented the assay with recombinant p47 (up to 0.5 \mu g/assay) alone or combined with p97, no increase in the signal was obtained (not shown). Inclusion of a monoclonal antibody against NSF (2E5) decreased by 50\% the \textsuperscript{35}S-GAG signal, and the inhibition was overcome by the presence of 0.5 \mu g of NSF (Fig. 5). Comparable levels of inhibition of intra-Golgi transport had been obtained with this antibody in the \textit{in vitro} transport assay developed by Rothman and co-workers (68). This antibody has also been found to inhibit transport (about 50\%) from the ER to the plasma membrane (69).

Rab proteins are a large family of GTP-binding proteins whose members are involved in many steps of intracellular transport (70–72). Rab function requires their association with membranes, and this step is coupled to nucleotide exchange (38, 73), a process controlled by the cytosolic protein guanine nucleotide dissociation inhibitor (Rab-GDI) (74). Addition of excess Rab-GDI will remove Rabs from membranes by locking them in their GDP-bound cytosolic form, thereby preventing their function in vesicular transport. This procedure has been reported to inhibit inter-cisternal Golgi transport (75, 76) as...
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Fig. 6. ARF and coatomer are involved in GAG synthesis in vitro. A mixture of 1.2 μg of purified rat coatomer plus 0.5 μg of recombinant myristoylated ARF was added to a standard GAGs in vitro assay in the presence or absence of 40 μM GTPγS. Lower two bars, cytosol (2 mg/ml) was omitted from the reaction. The mean ± S.E. of triplicate samples are shown in arbitrary units.

In addition to this evidence it would appear that COP-II vesicles do not take part in the Golgi synthesis of GAGs.

Golgi SNAREs Participate in Intra-Golgi Transport of GAGs—The current model to explain the specificity of vesicular transport (vesicles containing the right cargo fusing with the appropriate membrane) holds that vesicles will recognize their target membrane at the level of docking by controlling the pairing of cognate proteins present on the membranes that are to fuse. This idea arose from the identification of a family of proteins, present in vesicle and target membranes, capable of recognizing each other and able to bind crucial components of the fusion machinery, SNAPs (soluble NSF-attachment proteins). These proteins have been termed SNAREs (SNAP receptors) (85). Vesicle and target membrane SNAREs (v- and t-SNAREs) have been identified in different intracellular compartments. There is very strong evidence implicating SNAREs in many steps of membrane traffic (for reviews see Refs. 80, 86, and 87).

Several t- and v-SNAREs have been localized in the Golgi apparatus so far. It was obviously interesting to know whether these proteins would be involved in intra-Golgi transport, and therefore, we tried to address this question using the in vitro Golgi transport assay described in this study. The best known of Golgi SNAREs is syntaxin 5 (homologue of yeast Sed5), a t-SNARE localized mainly to the cis side of the Golgi complex (35, 88, 89) where it has been shown to be required for the forward transport of vesicles arising from the ER (88). Inclusion in our reaction mixture of 1 μg of an affinity purified polyclonal antibody raised against recombinant rat syntaxin 5 (35) potently inhibited synthesis of 35S-GAGs (Fig. 7A). A monoclonal antibody against a Golgi v-SNARE (GOS-28) also blocked GAG synthesis (i.e. transport) (Fig. 7A). GOS-28 had been previously shown to be required in ER to Golgi and cis-to medial-Golgi transport (90, but see Ref. 91). The block in intra-Golgi transport caused by the anti-syntaxin 5 antibody was unexpected as syntaxin 5 had been reported not to be involved in intra-Golgi transport (88). To substantiate this finding we prepared recombinant soluble syntaxin 5 and added it in increasing amounts to our reaction mixture. The prediction was that a true requirement of syntaxin 5 for Golgi transport would mean inhibition of the reaction after flooding the system with a SNARE unattached to membranes to compete with the endogenous, membrane-attached protein for the pairing with v-SNAREs. This is indeed what we found (Fig. 7B). Submicrogram amounts of purified recombinant syntaxin 5 inhibited the
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It is then clear that v- and t-SNAREs are indeed necessary for the intra-Golgi traffic of GAGs.

**DISCUSSION**

We present several lines of evidence that point to vesicular transport as being essential for GAG synthesis. First, there is an absolute requirement for transport conditions (ATP, cytosol, and physiological temperature); second, fragmentation of the Golgi apparatus during mitosis or under okadaic acid treatment (procedures known to arrest membrane transport) brings about a blocking of GAG biosynthesis; third, the process clearly requires the function of proteins involved in promoting membrane fusion (NSF) and in regulating docking (Rabs); fourth, synthesis utilizes proteins implicated in COP-I vesicle budding (ARF and coatomer); fifth, v- and t-SNAREs (GOS-28 and syntaxin 5), key components of the vesicle targeting machinery, are employed during the biosynthetic process.

This study reports the generation of sulfated GAGs onto an external acceptor in a cell-free system. In order to achieve this, we had to take particular note of the specific requirements for membrane transport, especially the presence of cytosol and ATP. It is at present unclear how many unitary transport steps are necessary to complete a single, sulfated xyloside-attached GAG chain because the exact localization of the biosynthetic reactions remains to be described and can only be, with the available data, tentatively allocated. The starting reaction (addition of the first Gal residue to xyloside) is believed to be an early Golgi event (88) although this could be the cis- or the medial-Golgi, and it is certainly separated from the next biosynthetic step (addition of the second Gal to Gal-Xyl) as shown in transport-arrested systems (10). The site of polymerization reactions is not clear-cut. Kinetic studies suggest it precedes sulfation (92, 93) although there is some evidence that sulfated GAGs can be further polymerized (94). However, sulfated GAGs are not formed if vesicular transport is stopped (this study and Refs. 12 and 18). The finding that GAG sulfation appears to be an all or nothing process, with some chains fully sulfated and others not sulfated at all (93), could be easily explained if there were separate compartments for polymerization and sulfation. Given the fact that GAG sulfation appears to be a trans-Golgi/TGN event (2, 18, 95), this would leave the polymerization reaction in the medial/trans compartment of the Golgi apparatus. Thus the completion of a GAG chain may be tentatively divided as follows: initiation, cisGolgi; polymerization, medial/trans-Golgi; and sulfation, trans/TGN. According to this view our in vitro system of GAG synthesis would require at least two inter-compartmental transport events. Nevertheless, the biosynthesis of a complete, sulfated GAG chain involving only one inter-compartmental transport step (separating, for instance, initiation from polymerization + sulfation) would still be compatible with the data presented in this study. Both the evidence we provide and that available in the literature cannot rule out this possibility.

The only cell-free intra-Golgi transport assay available until now is the well known system set up by Rothman and co-workers (96, 97) in which transport of VSV-G between cis- and medial-Golgi is measured. This assay has been extremely useful in unravelling the components involved in membrane transport. It requires, however, a mutant CHO cell line as well as virus-infected cells. Besides, only transport to the medial-Golgi is measured with the result that components specifically required for transport from the medial to the trans compartment would remain overlooked. An early report describing transport of VSV-G to the trans-Golgi in this cell-free system by measuring sialylation of VSV-G (98) has only rarely been used. It seems that biosynthetic transport of proteins from the ER to the trans/TGN can only be reliably reconstituted in permeabi-

**FIG. 7.** Syntaxin 5 and GOS-28 participate in the transport steps required for in vitro 35S-GAGs synthesis. A, the standard in vitro GAGs assay was supplemented with 1 μg of either polyclonal anti-syntaxin 5 (anti-Syn 5) or a monoclonal antibody against GOS-28 (anti-GOS-28). Net synthesis of 35S-GAGs is given as the mean ± S.E. of triplicate samples, and it is expressed as a percent of the control. B, standard reaction mixtures received the indicated amounts of recombinant soluble syntaxin 5 (p35). The amount of 35S-GAGs synthesized is given as a percent of the control. C, effect of addition of increasing quantities of antibody against syntaxin 5 (anti-Syn 5 (○)) to the in vitro GAGs assay. Addition of 1 μg of anti-Syn 5 plus 80 ng of recombinant syntaxin 5 (●) restores the 35S-GAGs synthesis to control levels.

synthesis of GAGs in a dose-dependent manner. In addition, low amounts of the recombinant protein reversed the inhibition brought about by the anti-syntaxin 5 antibody (Fig. 7C), stressing the specificity of the blocking.
lized cells. Attempts to detect sialylation of VSV-G with per-
ferated CHO cells were unsuccessful (99), but the system works well with perforated normal rat kidney cells (56). Nonetheless, identification of a component required for the last step in intra-Golgi transport would be difficult to achieve using the semi-intact cells approach if that component is also involved in ER to Golgi transport. The cell-free GAG assay we have de-
veloped in the present study should work with Golgi membranes isolated from other sources. This assay is absolutely linked to sialylation, a modification occurring in the most distal Golgi compartment. It might, therefore, allow the investigation and identification of components required for transport to the trans/ TGN compartment without the need for a previous obligatory ER to Golgi step indispensable in the protein transport systems using semi-intact cells.

The most surprising result of this study is our finding that syntaxin 5 is required for GAGs synthesis (i.e. intra-Golgi transport). Syntaxin 5 is a t-SNARE located in the cis-Golgi area that has been shown to be required for ER to Golgi traffic (88). As expected, it has been isolated in association with se-
veral v-SNAREs from the ER (100). From this evidence it would appear that syntaxin 5 is the target molecule recognized by incoming ER vesicles, an interpretation in line with the original formulation of the SNARE hypothesis (85). However, Sed5, the yeast analogue of mammalian syntaxin 5, functionally inter-
acts with v-SNAREs from other compartments such as Golgi Stfs (101) and Vti1p from the vacuole (102) indicating its participation in membrane transport events further along the secretory pathway. Some Sed5 mutants can, under certain conditions, accumulate invertase (a secretory protein) in an early Golgi compartment indicating that Sed5 might be re-
quired for some transport step to the distal Golgi (101). In agreement with this view, our finding of syntaxin 5 involve-
ment in 35S-GAGs synthesis could be explained if syntaxin 5 is required for medial- to trans/TGN traffic as blocking of this step would preclude sialylation. This effect could arise directly from a position of syntaxin 5 as a t-SNARE in a trans-Golgi compartment. Although the bulk of syntaxin 5 has been localized to the cis area of the Golgi complex, this does not rule out the presence of small amounts of syntaxin 5 in the trans/TGN side. Alternatively, syntaxin 5 in the cis-Golgi could be re-
quired for the arrival of recycling vesicles from distal Golgi compartments and would only be indirectly required in forward intra-Golgi transport. It is well established that mutants in which retrograde membrane traffic is defective are also af-
fected in forward transport (103, 104) presumably because of the need to recycle essentials components for anterograde movement. Both possibilities could explain our finding about the inactivation of GAG biosynthesis when syntaxin 5 function is impaired.

The in vitro GAG synthesis assay described in this paper provides an alternative system to follow intra-Golgi transport and should be useful to detect previously overlooked compo-
nents of this pathway such as syntaxin 5.

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