Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 9784–9788, December 1990
Cell Biology

Cell mutants defective in synthesizing a heparan sulfate proteoglycan with regions of defined monosaccharide sequence

(L cells/replica plating/heparan sulfate proteoglycans/antithrombin)

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Communicated by Phillips W. Robbins, August 8, 1990

ABSTRACT **We have demonstrated that mouse LTA cells synthesize cell-surface heparan sulfate proteoglycans (HSPGs) with regions of defined monosaccharide sequence that specifically interact with antithrombin (HSPG^{act}). It remains unclear how HSPG^{act} can be generated by a biosynthetic pathway with no simple template for directing the ordered assembly of monosaccharide units. To examine this issue, we treated LTA cells with ethyl methanesulfonate and then isolated seven stable mutants that synthesize only 8–27% of the wild-type HSPG^{act} but produce normal amounts of other HSPGs. These mutants are recessive in nature and fall into at least two different complementation groups. The delineation of the molecular basis of these defects should help to elucidate the manner by which cells synthesize HSPGs with regions of defined monosaccharide sequence.**

Mammalian cells synthesize heparan sulfate proteoglycans (HSPGs), which consist of core proteins with covalently linked glycosaminoglycans (GAGs) of 50–150 disaccharide units. The GAGs exhibit great structural diversity, which arises from differing arrangements of disaccharide units composed of alternating *N*-sulfate or *N*-acetylglucosamine residues with or without 6-*O*- and/or 3-*O*-ester sulfate groups and glucuronic acid or iduronic acid residues with or without 2-*O*-ester sulfate groups (1). It has been hypothesized that HSPGs may be involved in regulating the most basic aspects of cell biologic systems such as adhesion, proliferation, and differentiation (2, 3). However, considerable doubt exists about the specific nature of the above interactions because of a failure to isolate GAGs of unique monosaccharide sequence with appropriate biologic activities.

Recent advances in our knowledge of the structure–function relationships of the anticoagulant heparin have served as a model for elucidating the role of GAGs in more complex biologic systems. Proteolytic enzymes of the hemostatic mechanism are neutralized by forming a binary complex with a naturally occurring plasma protease inhibitor, antithrombin (AT) (4). Heparin preparations, which are a natural product of mast cells, contain about 30% GAGs, which bind tightly to AT and dramatically accelerate complex formation (5). These molecular species can only be isolated by affinity chromatography with the protease inhibitor. The GAGs that interact with AT exhibit a unique sequence of sulfated and nonsulfated uronic acid and glucosamine residues, which are required to complex with the protease inhibitor and induce a conformational change in the protein, that accelerates neutralization of blood coagulation enzymes (6–13).

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The highly specific interaction outlined above serves as the basis of a natural anticoagulant mechanism of the blood vessel wall. Endothelial cells synthesize HSPG of which only 1–10% exhibit heparan sulfate chains with appropriate monosaccharide sequences to bind to AT and accelerate neutralization of blood coagulation enzymes (HSPG^{act}) (14–16). The delineation of a specific biochemical function for an HSPG that possesses regions of defined monosaccharide sequence suggests that a similar situation may exist in many other biologic systems. However, it remains unclear how HSPG^{act} can be generated by a biosynthetic pathway with no simple template for directing the ordered assembly of different disaccharide units (1). One approach to this problem would be to isolate mutants that are defective in the synthesis of HSPG^{act} but possess no other gross defect in the overall production of HSPGs. This class of mutants might eventually allow the pinpointing of genes that regulate the monosaccharide sequence of GAGs. In this communication, we describe a cell line and screening technique that permits us to obtain mutants of this type.

MATERIALS AND METHODS

Materials. Electrophoretically homogeneous human AT was purchased from Cutter. *Flavobacterium* heparitinase was purified as described (14–16). Chondroitinase ABC, Pronase (protease type XIV), and papain (type IV) were obtained from Sigma. Carrier-free $Na_2{}^{35}SO_4$ was provided by ICN. All other chemicals used were of the highest grade available.

Iodination of AT. Human AT was radiolabeled with $Na^{125}I$ (^{125}I-AT; 15 mCi/μg; 1 Ci = 37 GBq; Amersham) by the chloramine-T method (17). During the iodination procedure, the heparin binding site of AT was protected by labeling the protease inhibitor in the presence of a 10-fold molar excess of affinity-fractionated octasaccharide (11).

Cells. Mouse LTA cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (GIBCO), 100 units of penicillin per ml, and 100 μg of streptomycin per ml (supplemented DMEM) in humidified 5% CO_2/95% air at 37°C. For metabolic labeling with $Na_2{}^{35}SO_4$, the cells were incubated in Na_2SO_4-free medium 199 supplemented with 10% fetal calf serum with no added antibiotics (supplemented medium 199).

Production and Isolation of LTA Mutants with Decreased Ability to Bind AT. Exponentially growing LTA cells were treated with 400 μg of ethyl methanesulfonate per ml in

Abbreviations: HSPG, heparan sulfate proteoglycan; HSPG^{act}, active antithrombin HSPG; GAG, glycosaminoglycan; AT, antithrombin; AMN, anhydromannitol.

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supplemented DMEM for 16 hr at 37°C. This method of mutagenesis was selected after determining the concentration of ethyl methanesulfonate and time of incubation with the alkylating agent that increased the frequency of the ouabain-resistance phenotype by 2–3 orders of magnitude (18).

Mutagenized LTA cells were then examined by a minor modification of the replica-plating technique of Raetz *et al.* (19). Cell colonies exhibiting decreased AT binding were identified, and mutant cells were isolated with glass cloning cylinders and trypsin and then were expanded in wells of increasing size. The mutant cells were then subjected to a second round of replica plating, and colonies with decreased AT binding but normal GAG synthesis were isolated and then cloned by limiting dilution in 96-well microtiter plates. Colonies derived from individual cells were assayed for AT binding, and mutant clones were grown for further study.

Characterization of GAG Chains by Enzyme Digestion, Affinity Chromatography, and Disaccharide Analysis. Confluent cell monolayers were incubated for 18–48 hr in Na_2SO_4-free supplemented medium 199 with 50 μCi of $Na_2{}^{35}SO_4$ per ml (characterization by enzyme digestion) or 650 μCi of $Na_2{}^{35}SO_4$ per ml (characterization by affinity fractionation and disaccharide analysis); washed with 150 mM NaCl/10 mM Na_2HPO_4, pH 7.4 (PBS); and treated with 0.05% trypsin/0.53 mM EDTA for 8 min at 24°C (characterization by enzyme digestion) or 20 min at 24°C (characterization by affinity chromatography and disaccharide analysis). The first technique reproducibly liberated 80–85% of the total labeled GAGs without significant cell lysis.

To characterize GAG chains by enzyme digestion, the above cell suspension was spun for 10 min at 400 × *g*. The supernatant was filtered through a 0.2-μm filter and dialyzed against 150 mM NaCl/50 mM Na_2HPO_4, pH 7.5, whereas the cell pellet was lysed in 2 M NaOH and assayed for protein content (20). The ^{35}S-labeled GAGs were then digested for 3 hr at 37°C with 0.16 units of *Flavobacterium* heparitinase or 0.2 units of chondroitinase ABC per ml or a combination of both enzymes. The resultant samples were examined by HPLC gel filtration on calibrated Spherogel TSK 2000SW (Beckman), and the fractions were assayed for ^{35}S.

To characterize GAG chains by affinity chromatography and disaccharide analysis, the above suspension was centrifuged for 20 min at 400 × *g*. The supernatant was treated sequentially with 0.1 mg of Pronase per ml for 18 hr at 37°C, 0.1 mg of Papain per ml for 18 hr at 37°C, and 0.01 unit of chondroitinase ABC per ml for 4 hr at 37°C. The heparan sulfate chains were isolated by filtering the material through a DEAE·Trisacryl column (Pharmacia; 2.5 cm × 40 cm) equilibrated with 150 mM NaCl/50 mM Na_2HPO_4, pH 7.4; washing the matrix with the above buffer until ^{35}S radioactivity dropped to background; eluting the contaminating proteins with two column volumes of 50 mM sodium acetate (pH 5.0); and harvesting the labeled GAGs with 1 M NaCl/50 mM Na_2PO_4, pH 7.4. The anticoagulantly active and anticoagulantly inactive fractions were obtained by affinity chromatography with AT and concanavalin A-Sepharose according to a minor modification of the technique of Jordan *et al.* (21). The heparan sulfate chains were deacetylated by hydrazinolysis and then subjected to high pH and low pH nitrous acid degradation as well as sodium borohydride reduction (16, 22, 23). After each chemical treatment, the samples were gel-filtered on a calibrated Bio-Gel P-4 column (0.8 cm × 190 cm) equilibrated in 0.5 M ammonium bicarbonate (pH 8.3), which revealed that the final products of the degradation process were disaccharides. These components were analyzed by HPLC ion-exchange chromatography on a Whatman Partisil 10 SAX column (16, 24).

RESULTS

Characterization of LTA Cells. The use of somatic cell genetic techniques to investigate the synthesis of $HSPG^{act}$ required a cell line that grows rapidly from clonal density, is easily manipulated to generate stable mutants, and produces large amounts of cell-surface $HSPG^{act}$. Several lines of evidence suggested that LTA cells, which had been previously utilized in somatic cell genetic analyses (25–27), synthesized cell-surface $HSPG^{act}$.

First, LTA cells produce heparan sulfate chains that interact specifically with AT and possess a protease inhibitor binding site sequence similar to that of anticoagulantly active heparin/heparan sulfate. To demonstrate that this was the case, LTA monolayers were metabolically labeled with $Na_2{}^{35}SO_4$, and heparan sulfate chains were isolated and then affinity-fractionated with AT and concanavalin A-Sepharose. In two separate experiments, an average of 5.5% of the heparan sulfate chains bound tightly to immobilized AT and was eluted with a high salt wash. The remaining heparan sulfate chains, with minimal affinity for the protease inhibitor, did not complex with immobilized AT and were found in the supernatant fraction. The nonspecific binding of radiolabeled heparan sulfate to the affinity matrix was <0.5%. The structural differences between the high-affinity heparan sulfate chains and those remaining in the supernatant fraction were established by quantitatively cleaving both populations of GAGs to disaccharides and then examining these species by ion-exchange HPLC. The average results of two separate experiments are summarized in Table 1. It is readily apparent that the high-affinity heparan sulfate chains are enriched with regard to GlcA-AMN-3-*O*-SO_3 and GlcA-AMN-3,6-*O*-SO_3, where AMN is anhydromannitol and GlcA is glucuronic acid, and that the depleted GAG chains possess minimal amounts of these disaccharides. This is to be expected because the two disaccharides are markers for the primary AT binding domain of heparin and heparan sulfate (6–13).

Secondly, the LTA cell surface exhibits significant amounts of $HSPG^{act}$ that can bind tightly to AT. To demonstrate the presence of these components, cell monolayers were incubated with various concentrations of ^{125}I-AT, and the levels of bound protease inhibitor were determined (Fig. 1). Inspection of the binding isotherm suggests that AT interacts with about 480,000 binding sites per cell with a K_d of about 5×10^{-8} M, which is similar to that previously obtained with cloned microvascular and macrovascular endothelial cells (14–16). To show that the protease inhibitor is bound specifically to cell-surface $HSPG^{act}$, cell monolayers were preincubated with purified *Flavobacterium* heparitinase, which virtually eliminates the subsequent binding of ^{125}I-AT (data not shown).

Autoradiographic Detection of Mutant Colonies with Defective AT Binding but No Gross Abnormalities of GAG Biosynthesis. The above results show that LTA cells synthesize $HSPG^{act}$ that is present on the surface of these cells. These

Table 1. Disaccharide analyses of heparan sulfate from LTA cell

Disaccharide	Heparan sulfate chains, %	
	Affinity-fractionated	Remaining
GlcA-2-*O*-SO_3 ⟶ AMN	1.5 ± 0.36	1.4 ± 0.21
GlcA ⟶ AMN-6-*O*-SO_3	13.4 ± 2.05	12.6 ± 0.87
IdA ⟶ AMN-6-*O*-SO_3	15.6 ± 1.75	21.9 ± 0.12
IdA-2-*O*-SO_3 ⟶ AMN	25.1 ± 2.69	31.5 ± 0.92
GlcA ⟶ AMN-3-*O*-SO_3	7.0 ± 1.04	0.2 ± 0.29
IdA-2-*O*-SO_3 ⟶ AMN-6-*O*-SO_3	32.7 ± 0.59	32.5 ± 1.10
GlcA ⟶ AMN-3,6-*O*-$(SO_3)_2$	4.1 ± 0.6	ND

GlcA, glucuronic acid; IdA, iduronic acid; AMN, anhydromannitol.

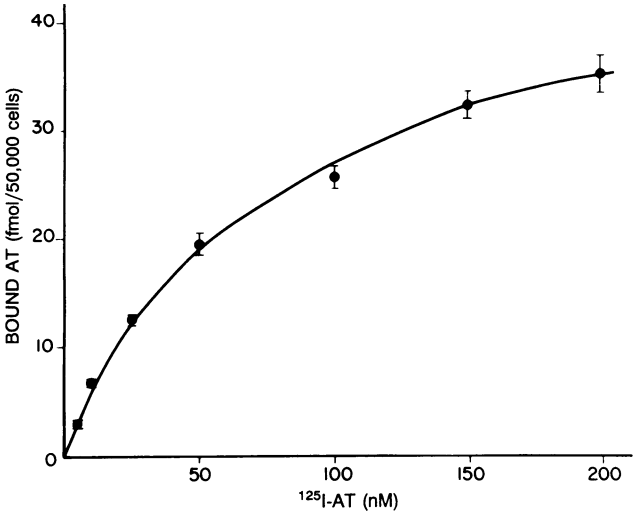

FIG. 1. Binding of ^{125}I-AT to LTA monolayers. The extent of binding of ^{125}I-AT to LTA cells was determined on confluent cell monolayers preincubated for 1 hr at 37°C with medium containing 1% Nutridoma SP (Boehringer Mannheim). The cell monolayers were then incubated for 1 hr at 4°C with various concentrations of ^{125}I-AT (average specific activity of 8×10^4 cpm/ng) diluted in PBS containing 1% Nutridoma SP and 50 μg of bovine serum albumin per ml with or without a 100-fold molar excess of unlabeled protease inhibitor. The cell monolayers were then washed with PBS containing 100 μg of bovine serum albumin per ml, and bound protease inhibitor was quantitated by removing cells with a cotton swab and subsequent assay of ^{125}I. Specific binding is calculated as the difference between binding in the presence and absence of a 100-fold molar excess of unlabeled protease inhibitor. Nonspecific binding averaged about 10% of specific binding. Cell numbers were estimated by trypsinizing control wells and then counting cells on a Coulter Counter. All determinations were performed in triplicate. The mean values and error bars represent the results of one typical experiment with associated standard errors. Similar results were obtained in two other independent experiments.

observations suggested that this proteoglycan could be detected *in situ* in LTA colonies immobilized on polyester cloth. In preliminary experiments, we demonstrated that LTA cells are able to bind ^{125}I-AT as judged by autoradiography in proportion to (*i*) the protein content of the colony as quantitated by Coomassie staining intensity or (*ii*) the GAG content of the colony as determined by incorporation of $^{35}SO_4^{2-}$. To generate mutants with decreased ability to synthesize HSPG^{act}, LTA cells were treated with ethyl methanesulfonate and then seeded at clonal density for screening on replica filters to evaluate AT binding. The mutagen-treated colonies yielded an occasional variant that failed to complex with the protease inhibitor. Putative mutants identified in this way were harvested and then were passed through a second round of screening to enrich their numbers and to confirm their altered phenotypes (Fig. 2*A*).

The above colonies were also screened for their ability to incorporate $^{35}SO_4^{2-}$, since our goal was to isolate mutants that exhibited restricted defects in the synthesis of HSPG^{act} but possessed no gross abnormalities in GAG biosynthesis (Fig. 2*B*). As shown for mutant VI-51, replica filters obtained during the second round of screening exhibited interspersed wild-type and mutant colonies with regard to AT binding normalized to Coomassie staining, but both types of colonies possess the same intensity of $^{35}SO_4^{2-}$ incorporation normalized to Coomassie staining. All other isolated mutants showed the same characteristics with regard to AT binding and $^{35}SO_4^{2-}$ incorporation. The mutants were finally cloned by limiting dilution and exhibited a homogenous population of colonies with regard to minimal AT binding as evaluated on replica filters. The screening of 40,000 colonies yielded

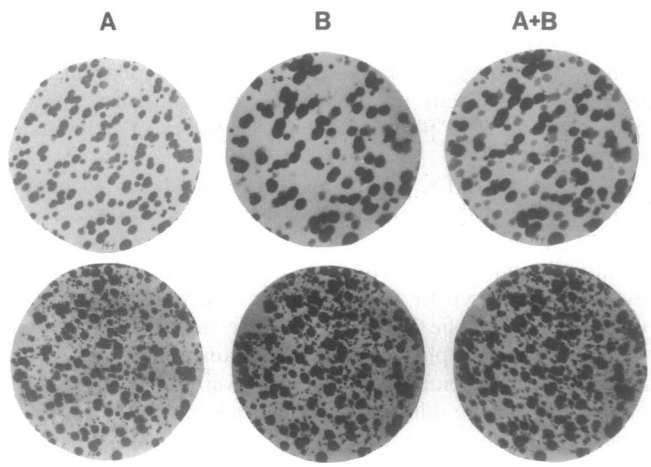

FIG. 2. Second round of replica filter analyses of mutant VI-51 as judged by AT binding and synthesis of GAGs. The duplicate replica filters for the second round of screening of mutant VI-51 are depicted with respect to AT binding (top filters) and GAG biosynthesis (bottom filters). The filter assay for AT binding was carried out with cells grown on replica filters. The technique used for AT binding is identical to that described in Fig. 1 except that the concentration of ^{125}I-AT was set at 0.2 nM, filters were constantly agitated, and bovine serum albumin was omitted in the final wash. Upon completion of the above procedure, cell colonies were fixed, stained with Coomassie brilliant blue (2 g/liter) dissolved in MeOH/CH_3COOH/H_2O, 4.5:1:4.5 (vol/vol), destained in the same solvent mixture, and air-dried. The filter assay for GAG synthesis was conducted as outlined for the labeling of cell-surface glycoconjugates except that filters were incubated with 50 μCi of $Na_2{}^{35}SO_4$ per ml for 24 hr, washed with ice-cold 10% (vol/vol) CCl_3COOH, air-dried, and stained with Coomassie brilliant blue as outlined above. The stained filters were exposed to autoradiography using Kodak X-OMAT AR films (Eastman Kodak) with one Cronex Xtra Life intensifier screen (DuPont) at −70°C for 18–24 hr and then were developed by using a Kodak RP X-OMAT processor. (*A*) Coomassie staining of duplicate filters for the second round of screening of mutant VI-51. (*B*) Autoradiographs of ^{125}I and ^{35}S cpm on the same replica filters. (*A*+*B*) Superimposition of the autoradiographs and Coomassie-stained filters.

seven mutant lines, each derived from five separate mutagenized cell populations. These mutants exhibited stable phenotypes for a minimum of 6 months in culture and possessed no obvious alterations with regard to gross morphology, trypsin sensitivity, or adhesion.

Characterization of Mutants. We wished to determine the extent to which the various mutants are able to synthesize cell-surface HSPG^{act}. The data revealed that the mutants exhibited a residual ability to bind AT that ranged from 8% to 27% of the wild-type cells when protease inhibitor concentrations of 10 nM were utilized (Fig. 3). Similar results were apparent at AT concentrations of 50 nM and 200 nM, respectively (data not shown). Additional studies were carried out that showed that the extent of HSPG^{act} shedding into the medium is proportional to the amount of cell-surface HSPG^{act} present on the various mutant cell lines (data not shown). The heparan sulfate chains were also isolated from several of the mutants, and affinity fractionation with AT revealed that <0.5–1.0% of the labeled GAGs bound to the protease inhibitor. Therefore, the observed reductions in AT binding by the mutants are caused by a restricted defect in the synthesis of HSPG^{act} and not by a general reduction of cell-surface HSPG.

We attempted to ascertain whether the various mutants exhibited alterations in overall GAG biosynthesis. The previous observation of identical incorporation of $^{35}SO_4^{2-}$ by mutant and wild-type LTA cell colonies as judged by replica plating suggests that the two types of cells possess a quali-

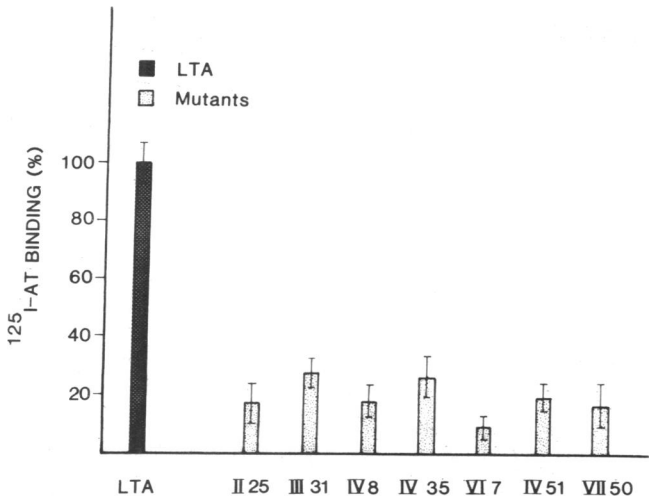

FIG. 3. Binding of AT to wild-type and mutant LTA cell monolayers. The binding of ^{125}I-AT to wild-type and mutant LTA cell monolayers was determined as outlined in Fig. 1 except that the protease inhibitor concentration was 10 nM. The mean values and error bars represent the results of three to five separate experiments with associated standard errors. Mutant cell lines were cultured continuously for up to 6 months prior to assay.

tatively similar capacity to produce GAGs. To detect biosynthetic abnormalities in a more definitive fashion, we incubated the various cell lines with $Na_2{}^{35}SO_4$ under conditions that label GAGs to constant specific activity, quantitatively released the glycoconjugates with trypsin, and then determined the amounts of chondroitin sulfate and heparan sulfate harvested relative to cell protein. The extent of $^{35}SO_4^{2-}$-labeled material released from wild-type LTA cells averaged 1.2×10^6 cpm/mg of cell protein ($n = 3$), whereas the same parameter in the seven mutant cell lines ranged from 0.9×10^6 cpm/mg of cell protein to 1.6×10^6 cpm/mg of cell protein. The labeled GAGs were then characterized by enzymatic degradation with *Flavobacterium* heparitinase and/or chondroitinase ABC and subsequent quantitation of the digested products by HPLC gel filtration. The above analyses were carried out for wild-type LTA cells, which showed that 70 ± 4% of the labeled material was degraded to tetrasaccharides/disaccharides with *Flavobacterium* heparitinase ($n = 4$), whereas 32 ± 4% of the labeled material was cleaved to the same size fragments with chondroitinase ABC ($n = 4$). Similar studies conducted with the seven mutant cell lines showed that 63–80% of the labeled material is degraded to tetrasaccharides/disaccharides with *Flavobacterium* heparitinase, whereas 20–37% of the labeled material is cleaved to the same size fragments with chondroitinase ABC. Heparan sulfate and chondroitin sulfate chains of the wild-type and mutant cells exhibited the same approximate molecular sizes as judged by HPLC gel filtration. Thus, it would appear that the seven mutant cell lines with defective binding of ^{125}I-AT possess the ability to synthesize normal amounts and sizes of heparan sulfate and chondroitin sulfate chains.

We also initiated investigations to determine the numbers of different mutations present within our collection of mutants. To this end, we carried out complementation analyses on two of our mutants and the wild-type cells. Pairs of mutants and wild-type cells were fused by treating mixed cell monolayers with polyethylene glycol, the resulting cell populations containing some hybrids were plated at high density on filters, and the frequency of hybrids able to bind ^{125}I-AT was evaluated by autoradiography. Fig. 4 depicts the results obtained after fusing wild-type LTA cells with themselves (positive control), mutant VI-7 and mutant VII-50 with themselves (negative controls), and mutant VI-7 with mutant

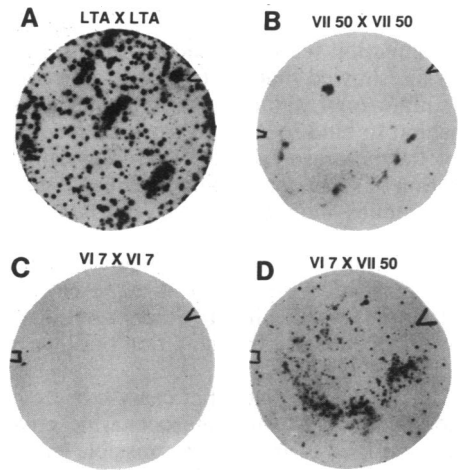

FIG. 4. Complementation analysis of mutants VI-7 and VII-50. The various wild-type or mutant cell pairs were seeded on tissue culture dishes at a density of 10^5 cells per cm^2. After 24 hr, the cells were washed with DMEM, fused by incubating for 1 min with 50% polyethylene glycol (PEG 1450, Kodak), and washed again as described above. The cells were fed with DMEM supplemented with 10% fetal calf serum containing antibiotics and then were allowed to recover for 24 hr. The total cell populations were trypsinized, 10^4 cells from each fusion were directly seeded on polyester 17-μm mesh filters, and the cells were allowed to grow for 12 days to generate about 500 individual colonies per filter. The various filters were then assayed for AT binding and Coomassie staining as described in Fig. 2. The autoradiographs of the filters for the various fused cell populations are provided. (*A*) Fusion of LTA cells with LTA cells. (*B*) Fusion of mutant VII-50 with mutant VII-50. (*C*) Fusion of mutant VI-7 with mutant VI-7. (*D*) Fusion of mutant VI-7 with mutant VII-50.

VII-50. It is readily apparent that self-fusion of VI-7 generates hybrids that cannot bind AT and that self-fusion of VII-50 produces a similar picture except for rare blotchy areas of residual uptake of protease inhibitor. It is clearly evident that fusion of VI-7 with VII-50 as compared with the self-fusion of the same mutants generates a large number of small colonies that are able to bind AT as well as the self-fusion of wild-type cells. Examination of the same filters by Coomassie staining indicated no significant differences between the various cell populations except for a dramatically increased cell density in regions that correspond to the rare areas of residual uptake of protease inhibitor by the self-fusion of VII-50 (data not shown). Thus, the above data suggest that the mutations that lead to VI-7 and VII-50 are recessive in nature and belong to two different complementation groups.

DISCUSSION

The synthesis of HSPG^{act} requires the production of a core protein; assembly of a linkage region of four monosaccharide units coupled to specific serine residues; generation of a precursor oligosaccharide chain of alternating *N*-acetylglucosamine and glucuronic acid residues; and subsequent remodeling of this repetitive structure by variable epimerization of glucuronic acid residues, variable sulfation of the glucuronic and iduronic acid residues, and variable N-deacetylation and N-resulfation of glucosamine resides in conjunction with variable 6-*O*- and 3-*O*-ester sulfation (1). The end result of this complex process is the generation of a region of unique carbohydrate structure that at the very least contains the monosaccharide sequence: nonsulfated uronic acid-[*N*-acetyl(*N*-sulfate)glucosamine 6-*O*-sulfate]-glucuronic acid-[*N*-sulfate glucosamine 3-*O*-sulfate (6-*O*-sulfate)]-iduronic acid 2-*O*-sulfate–[N-sulfate glucosamine 6-*O*-sulfate

(6–13)]. The use of natural and synthetic fragments of heparin, in conjunction with fast-reaction kinetics and equilibrium dialysis, showed that the 6-*O*-sulfate on residue 2 and the 3-*O*-sulfate on residue 4 act in a thermodynamically linked fashion to bind to AT and trigger the conformational change of the protease inhibitor that is essential for rapid neutralization of some coagulation enzymes, whereas sulfate groups at residues 5 and 6 dramatically augment interaction with the protease inhibitor (12, 13). Carboxyl groups on uronic acid residues within this sequence may also be of biologic importance. Finally, it is apparent that regions outside the primary AT binding domain outlined above are essential for accelerating the inhibition of other blood coagulation enzymes (28, 29).

The biosynthetic enzymes that carry out the posttranslational modifications of the oligosaccharide chains are not available in purified form, with the possible exception of the *N*-sulfotransferase (30), and little is known about how these enzymes are coordinately regulated within the Golgi complex. It has been postulated that the synthesis of heparan sulfate chains with regions of defined monosaccharide sequence might be achieved by specific interactions between core protein regions and multimolecular arrays of biosynthetic enzymes/modulating proteins within the Golgi complex or alternatively by the random action of early biosynthetic enzymes with restricted substrate preference of later biosynthetic enzymes for certain subsets of the randomly generated monosaccharide sequences. Resolution of this issue is important to our understanding of how cells might synthesize $HSPG^{act}$ with regions of defined monosaccharide sequence and whether GAGs are likely to modulate complex biologic systems via specific interactions with target molecules.

We have attempted to establish a somatic cell genetic system for investigating the synthesis of $HSPG^{act}$ that may be helpful in resolving the above question. To this end, we demonstrated that LTA cells synthesize cell-surface $HSPG^{act}$, isolated stable mutants of this cell type that are defective in the synthesis of the unique monosaccharide sequence required to bind AT but have no gross alterations in the overall pathways of GAG biosynthesis, and showed that the above mutations are due to alterations in at least two different genes. Our overall approach to this problem is based upon the pioneering studies of Esko and coworkers who mutagenized CHO cells that synthesized GAG chains with no ability to bind to AT and identified mutants in the GAG biosynthetic pathway by replica-plating methods involving $^{35}SO_4^{2-}$ incorporation (18, 31–33). These investigators were able to isolate at high frequency a variety of mutants with reduced ability to transport the SO_4^{2-} ion, to synthesize the linkage region required for coupling of heparan sulfate or chondroitin sulfate chains to core proteins, or to sulfate *N*-glucosamine residues. The isolation of the above mutants at high frequency clearly indicates that the GAG biosynthetic pathway may be amenable to dissection by genetic approaches. It is of interest to note that our investigations also document a relatively high frequency of mutation with regard to the biosynthetic pathways needed to synthesize the AT binding site, but none of our mutations is caused by alterations in SO_4^{2-} transport or a decreased ability to generate the linkage region as described above. This is most likely due to the use of different screening approaches to identify our mutants.

At the present time, we have no detailed knowledge of the molecular basis of our mutants except that none exhibits a defect in the GAG chain elongation mechanism. It is possible that alterations have been directly or indirectly induced in core protein(s), *N*- or *O*-sulfotransferase enzymes, or potential modulatory proteins involved in establishing monosaccharide sequence. The ongoing investigations of the detailed structure of the heparan sulfate chains of the mutants and direct enzyme assays of the various steps in the biosynthetic pathway of the mutants should pinpoint the abnormalities. The correlation of these changes, including reductions in the levels of *N*- or *O*-sulfotransferases, with the loss of AT binding sites should greatly improve our understanding of how cells generate HSPG with regions of defined monosaccharide sequence.

It is a pleasure to acknowledge helpful discussions with Drs. James Marcum, Tet Kojima, and Joseph Miller. We thank Dr. Monty Kreiger for a critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (HL 33014) and the National Foundation for Cancer Research and by fellowship funds from Glycomed Inc., San Francisco, CA.

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