

Correlation between Cell Substrate Attachment In Vitro and Cell Surface Heparan Sulfate Affinity for Fibronectin and Collagen

STAMATIS C. STAMATOGLOU and JOHN M. KELLER

Department of Biochemistry, University of Health Sciences/The Chicago Medical School, North Chicago, Illinois 60064

ABSTRACT Heparan sulfate glycosaminoglycan, isolated from the cell surface of nonadhering murine myeloma cells (P3X63-Ag8653), does not bind to plasma fibronectin, but binds partially to collagen type I, as assayed by affinity chromatography with proteins immobilized on cyanogen bromide-activated Sepharose 4B. Identical results were obtained when myeloma heparan sulfate was co-chromatographed, on the same fibronectin and collagen columns, with cell surface heparan sulfates from adhering Swiss mouse 3T3 and SV3T3 cells. These latter heparan sulfates do, however, bind to both fibronectin and collagen, as reported earlier (Stamatoglou, S. C., and J. M. Keller, 1981, *Biochim. Biophys. Acta.*, 719:90-97). Cell adhesion assays established that hydrated collagen substrata can support myeloma cell attachment, but fibronectin cannot. Saturation of the heparan sulfate binding sites on the collagen substrata with heparan sulfate or heparin, prior to cell inoculation, abolished the ability to support cell adhesion, whereas chondroitin 4 sulfate, chondroitin 6 sulfate, and hyaluronic acid had no effect.

Cell surface heparan sulfate is a ubiquitous cell surface proteoglycan (1) believed to be attached to the plasma membrane via a hydrophobic region in the proteoglycan core protein (2-4). Enrichment of cell-substrate adhesion sites with heparan sulfate (5, 6) and binding of cell surface heparan sulfate to extracellular adhesive proteins such as fibronectin (7, 8) and collagen (8) suggest an involvement in cell adhesion. On the basis of this suggestion we predict that some nonadhering cells might either lack this cell surface molecule or have structurally altered cell surface heparan sulfate with no affinity for adhesive matrices. In this study we used nonadhering myeloma cells to demonstrate a correlation between cell surface heparan sulfate affinity for collagen or fibronectin and cellular attachment to collagen or fibronectin substrata. That these cells do not adhere in vitro facilitated our adhesion assays since no enzymatic treatment (which would remove cell surface molecules) was required to produce single-cell suspensions. Furthermore, plasma cells are not known to produce any extracellular collagenous matrices, which could interfere with the results.

MATERIALS AND METHODS

Materials: Radioisotopes ($H_2^{35}SO_4$, D-[1- ^{14}C]glucosamine, D-[6-(N) 3H]glucosamine), cyanogen bromide (CNBr)-activated Sepharose 4B, Hepes, collagen, reference standard chondroitin 4 sulfate, chondroitin 6 sulfate, heparin, and hyaluronic acid were obtained from sources mentioned in an earlier report (8). [Methyl- 3H]thymidine was purchased from New England Nuclear, Boston, MA. Heparan sulfate, a gift from Dr. K. L. Keller (Chicago Medical School, North Chicago, IL), was purified from a crude heparan sulfate preparation from Upjohn Co. (Kalamazoo, MI) according to established procedures (9, 10).

Cell Cultures: The P3X63-Ag8653 line of murine myeloma cells was obtained from the Institute of Medical Research (Camden, NJ) and maintained in Dulbecco-Vogt medium (11), supplemented with 10% fetal bovine serum. The growth conditions for the Swiss mouse 3T3 and SV3T3 cells used in this laboratory have been described (12, 13).

Isolation of Radiolabeled Heparan Sulfate Glycosaminoglycans: Cell surface heparan sulfate was isolated from 3T3, SV3T3, and myeloma cells metabolically radiolabeled with D-[1- ^{14}C]glucosamine, D-[6-(N) 3H]glucosamine, or $H_2^{35}SO_4$. The preparation protocols (12, 14) use DEAE-cellulose ion exchange chromatography to isolate the glycosaminoglycans from pronase-digested cell surface trypsin extracts. The purified heparan sulfate is isolated on a second DEAE-cellulose column after degradation of contaminating chondroitin sulfate with chondroitin ABC lyase. Radiochemical purity of heparan sulfate was confirmed by nitrous acid degradation (1, 15). The myeloma heparan sulfate glycosaminoglycan has a lower molecular weight, iduronic acid content, *N*-*O*-sulfate ratio, and charge density than either the 3T3 or SV3T3 heparan sulfate (16).

Fibronectin Isolation: Fibronectin was isolated from human plasma on a gelatin/CNBr-activated Sepharose 4B affinity column (17). Purity was confirmed by polyacrylamide slab gel electrophoresis (18).

Affinity Chromatography: Affinity chromatography of heparan sulfate on protein-linked CNBr-activated Sepharose columns was performed as previously described (8). The running buffer was 10 mM Hepes, and 150 mM NaCl (Hepes-saline), pH 6.50. Elution of bound material was effected with 2 M NaCl in the running buffer. As in previous experiments (8), the recovery of radio-labeled material was >95%.

Preparation of Cell Culture Substrata: All substrata were prepared on 35 × 10-mm Falcon tissue culture dishes (Falcon Labware, Oxnard, CA). The preparation of hydrated collagen substrata was based on a published method (19). A 3-mg/ml solution of calf skin collagen in 0.1% acetic acid was mixed with an equal volume of twice concentrated Hepes-saline and neutralized to pH 7.00. 700- μ l aliquots of this solution were immediately dispensed into culture dishes and incubated at 37°C for 40 min in a humidified incubator. Under these conditions collagen formed a lightly translucent gel. Its native

conformation was confirmed by identifying collagen fibrils with scanning electron microscopy of critical point-dried samples.

Collagen substrata were treated with glycosaminoglycans (100 µg/dish for 30 min at 37°C) in 1 ml of adhesion buffer (Hepes-saline, pH 6.50, 0.1% glucose, and 0.1% bovine serum albumin). This solution was then removed and 1 ml of adhesion buffer alone was added immediately prior to cell seeding. In preliminary studies we incubated collagen substrata with heparin solutions of various concentrations, and by uronic acid analyses (20) of nonbound heparin we established that 100 µg of heparin was sufficient to saturate the heparin-binding sites on the collagen. The collagen-coated dishes used for controls to glycosaminoglycan treatments were treated identically in the absence of glycosaminoglycan. Fibronectin-coated dishes were prepared by a 30-min incubation (37°C) of dishes with 1 ml of adhesion buffer containing 100 µg fibronectin per dish. Dishes treated in this manner supported attachment of SV3T3 cells in adhesion buffer and induced enhanced spreading of the same cells in serum-supplemented culture medium. Control dishes were incubated with 1 ml of adhesion buffer.

Adhesion Assay: Myeloma cells seeded at a density of 10⁵ cells/ml were labeled after 24 h with [methyl-³H]thymidine (10 µCi/ml). The cells were grown in the presence of the isotope for another 20 h. Prior to the experiment the cells were collected by centrifugation, washed twice with Hepes-saline, and resuspended in adhesion buffer. Cells were seeded to the appropriately treated dishes at a density of 10⁵ cells per 35 × 10 mm-dish, and the dishes were placed in an humidified incubator (adhesion at zero time was measured immediately after inoculation, i.e., without incubation). At specified intervals (10, 30, 60, 90, and 120 min) the nonattached cells were harvested from each dish by collecting the medium and rinsing each dish twice with Hepes-saline, and dissolved in Budget Solve (Research Products International Corp., Mt. Prospect, IL). Adherent cells were dissolved in situ with 1 ml of 5% SDS and transferred to Budget Solve. All samples were counted in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). The radioactivity remaining on the dish, expressed as a percentage of the total recovered radioactivity associated with adhering plus nonadhering cells, reflects the percentage of attaching cells. To preserve objectivity, duplicate experiments were carried out by different persons and the labels were coded.

RESULTS

Interactions of Myeloma Cell Surface Heparan Sulfate with Fibronectin and Collagen

Cell surface heparan sulfate isolated from myeloma cells was co-chromatographed with 3T3 and SV3T3 heparan sulfates on fibronectin and collagen immobilized on CNBr-activated Sepharose 4B. 3T3 and SV3T3 heparan sulfates bound to both fibronectin and collagen (Fig. 1; [8]), whereas myeloma heparan sulfate bound only to collagen (Fig. 1b). Lack of affinity for fibronectin and a ~35–45% binding to collagen were consistently demonstrated in repeated experiments using two different preparations of myeloma cell surface heparan sulfate. We observed no differences on these columns between myeloma heparan sulfate alone and myeloma heparan sulfate together with heparan sulfates from either 3T3 or SV3T3 cells. The partial binding of myeloma heparin sulfate to collagen indicated a heterogeneous molecular composition, and this was confirmed by re-chromatography of the collagen-binding fraction on collagen/Sepharose: Repeated binding could be demonstrated (Fig. 1c). The small amount of myeloma heparan sulfate adhering to the fibronectin/CNBr-activated Sepharose 4B column was attributed to nonspecific interactions whose level (10%) was determined after chromatography of heparan sulfates on CNBr-activated Sepharose 4B derivatized in the absence of protein.

Interaction of Myeloma Cells with Fibronectin and Collagen Substrata In Vitro

Attachment of myeloma cells to the hydrated collagen-coated dishes, but not to fibronectin-coated dishes, was observed (Fig. 2). We followed the kinetics of adhesion for 120 min. Most of the myeloma cells attached to the collagen

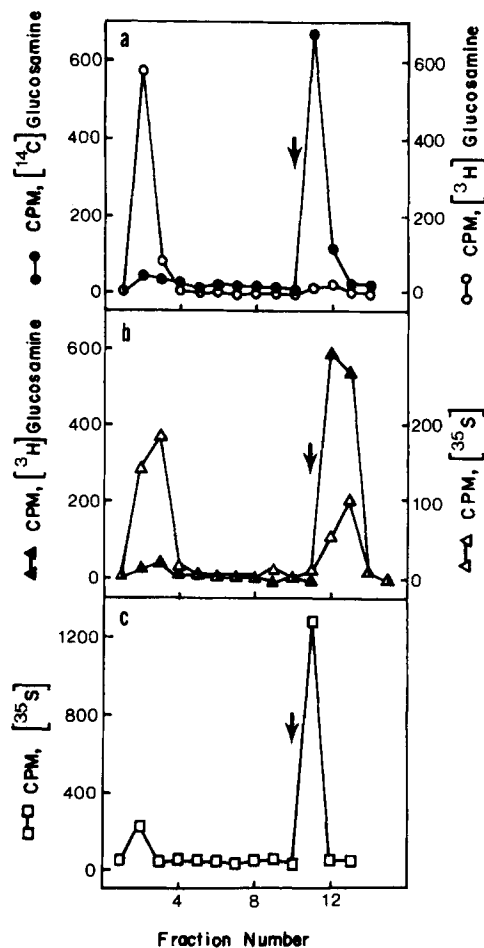


FIGURE 1 Co-chromatography of myeloma cell surface heparan sulfate with 3T3 or SV3T3 heparan sulfate on fibronectin or collagen-linked CNBr-activated Sepharose 4B (a and b) and re-chromatography of the collagen-binding fraction of heparan sulfate on collagen/Sepharose (c). Samples were applied at fraction 1 in 10 mM Hepes containing 0.150 M NaCl, pH 6.50. The beginning of elution, with 2 M NaCl in the same buffer, is indicated by the arrow. Fractions of 2 ml were collected throughout. (a) [³H]Glucosamine-labeled myeloma heparan sulfate (○) chromatographed with, [¹⁴C]glucosamine-labeled 3T3 heparan sulfate (●) on fibronectin/Sepharose. (b) [³⁵S]Sulfate-labeled myeloma heparan sulfate (△) and [³H]glucosamine-labeled SV3T3 heparan sulfate (▲) chromatographed on collagen/Sepharose. (c) Collagen-binding fraction of ³⁵S-labeled myeloma heparan sulfate (□) re-chromatographed on collagen/Sepharose column.

substrata within 10 min after inoculation, whereas adhesion to fibronectin-coated and control dishes remained close to background level (adhesion at zero time; Fig. 2). Examination of the adhering cells with light microscopy indicated that the cells retained their spherical shape, i.e., no spreading was detected.

Effect of Reference Standard Glycosaminoglycans on Myeloma Cell Adhesion to Collagen Substrata

In these experiments we examined the effect of saturating the heparan sulfate binding sites of the collagen substrata prior to the cell attachment assays. Reference standard heparan sulfate and heparin were used for this purpose and their effect on cell adhesion is shown in Table I. Both glycosaminoglycans

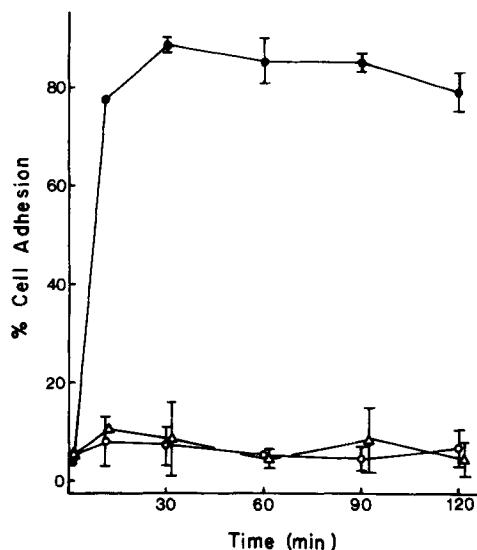


FIGURE 2 Adhesion assay of myeloma cells on pretreated cell culture dishes. Myeloma cells labeled with [³H]thymidine were suspended in adhesion buffer (10 mM Hepes, 150 mM NaCl, pH 6.50, containing 0.1% glucose and 0.1% bovine serum albumin) and plated on dishes preincubated with collagen (●), fibronectin (Δ), or adhesion buffer alone (○). The actual method of substrata preparation and cell harvesting is described in Materials and Methods. The percentage of total radioactivity remaining on the culture dish is expressed as percentage of attaching cells.

reduced the number of adhering cells by >90%. To demonstrate specificity of inhibition we incubated collagen substrata with reference standard chondroitin 4 sulfate and chondroitin 6 sulfate (each of which possesses a charge density similar to that of heparan sulfate) and with hyaluronic acid. None of these glycosaminoglycans significantly affected cell attachment (Table I).

DISCUSSION

Myeloma cells and a sizable fraction of myeloma cell surface heparan sulfate bind to collagen but not to fibronectin. Saturation of the heparan sulfate binding sites on the collagen with heparan sulfate (or heparin) inhibited cell attachment to these collagen substrata. The data indicate that the myeloma cell surface receptor for adhesion-promoting collagen is heparan sulfate. Relevant to our findings is that some cell types are known to respond to collagen-mediated adhesion in the absence of fibronectin (19, 21, 22). In addition, inhibition of cell attachment to substrate-attached material (SAM; [23]) can be induced by preincubating this material with heparan sulfate or heparin (24).

Our study corroborates accumulated evidence on cell surface heparan sulfate involvement in fibronectin-mediated adhesion of mesenchymal cells to extracellular matrices (5–8, 24): The heparan sulfate from the nonadhering myeloma cells does not bind to fibronectin nor can fibronectin support adhesion of these cells. However, at least in some cell types, the primary adhesion-determining cell receptor for fibronectin may not be heparan sulfate: A fibronectin domain that assures attachment of normal rat kidney cells *in vitro* does not contain the heparan sulfate binding site (25). Additionally, a recent report has demonstrated that heparin does not block the attachment to or spreading on fibronectin by murine SVT2 cells (26). Interestingly, although treatment of BALB c/3T3 cells with heparinase

TABLE I
Glycosaminoglycan Inhibition of Cell Attachment to Collagen Substrata

Time after inoculation min	Glycosaminoglycan				
	Heparan sulfate	Heparin	Chondroitin 4 sulfate	Chondroitin 6 sulfate	Hyaluronic acid
30	96	94	0	2	4
60	87	93	0	3	0
90	95	97	0	10	1
120	92	97	0	0	0

Radiolabeled myeloma cells were seeded on hydrated collagen substrata pretreated with glycosaminoglycans (see Materials and Methods). The percent inhibition of adhesion was calculated from the percent of adhering cells (i.e., percent of total radioactivity associated with attaching cells) on glycosaminoglycan-treated collagen substrata and on plain collagen substrata.

under conditions that removed >85% of the cell surface heparan sulfate did not prevent attachment to fibronectin, the spreading of these cells was greatly retarded, suggesting that the cell surface heparan sulfate is essential for the full spreading response of these cells (26). The cell surface fibronectin receptors in these latter cases could be gangliosides (27) or a group of glycoproteins with $M_r \sim 140,000$ (28, 29).

Cell surface heparan sulfates are structurally diverse molecules, and this diversity is related to the species and organ of origin (30) and to transformation (12, 14). The latter is accompanied by a decrease in the *O*- to *N*-sulfate ratio (15, 31), which does not seem to affect the heparan sulfate affinity for either fibronectin or collagen (8). In this report, however, a novel structure-function relationship is demonstrated. Heparan sulfate from the surface of nonadhering myeloma cells does not bind to fibronectin and is grossly different in structure compared with the fibronectin-binding heparan sulfates from adhering 3T3 and SV3T3 cells: Myeloma heparan sulfate ($M_r = 25,000$) is almost one-tenth the size of either 3T3 or SV3T3 heparan sulfate, and its iduronic acid content, *N*- to *O*-sulfate ratio, and charge density are, also, much lower (16). So far, our studies indicate that *N*-sulfate and/or iduronic acid content may be important parameters in the heparan sulfate/fibronectin interactions (16). Such interactions may be important not only in adhesion but also in forming extracellular matrices (32, 33).

In conclusion, we have shown that cell surface heparan sulfate can attach cells to collagen fibers. Similarly, other investigators have recently shown that cell surface heparan sulfate mediates attachment to a substrata of platelet factor 4 (26). Our data also indicate a similar role for heparan sulfate in fibronectin-mediated adhesion, but this is a moot issue now in view of conflicting evidence. The myeloma heparan sulfate we used herein is the first reported heparan sulfate with no affinity for fibronectin. This observation serves to underline the heterogeneity of heparan sulfates and their potential importance in a variety of cellular functions.

We thank Dr. K. L. Keller for providing a sample of reference standard heparan sulfate, Drs. L. J. Lowe-Krentz, K. L. Keller, M. Hosey, and R. Silver for helpful comments, Irene Stavros for technical assistance, and Mary Jo Lindgren for typing the manuscript.

The work was supported by grants HD 11770 and CA 23016 from the National Institutes of Health.

Received for publication 17 December 1982, and in revised form 28 February 1983.

REFERENCES

1. Kraemer, P. M. 1971. Heparan sulfates of cultured cells. II. Acid-soluble and -precipitable species of different cell lines. *Biochemistry*. 10:1445-1451.
2. Kjellén, L., I. Pettersson, and M. Höök. 1981. Cell-surface heparan sulfate: an intercalated membrane proteoglycan. *Proc. Natl. Acad. Sci. USA*. 78:5371-5375.
3. Norling, B., B. Glimelius, and Å. Wasteson. 1981. Heparan sulfate proteoglycan of cultured cells: demonstration of a lipid- and a matrix-associated form. *Biochem. Biophys. Res. Commun.* 103:1265-1272.
4. Lowe-Krentz, L. J., and J. M. Keller. 1981. The core proteins of heparan sulfate proteoglycans from Swiss mouse 3T3 cells. *Fed. Proc.* 40:1840.
5. Culp, L. A., B. J. Rollins, J. Buniel, and S. Hitri. 1978. Two functionally distinct pools of glycosaminoglycan in the substrate adhesion site of murine cells. *J. Cell Biol.* 79:788-801.
6. Rollins, B. J., and L. A. Culp. 1979. Glycosaminoglycans in the substrate adhesion sites of normal and virus-transformed murine cells. *Biochemistry*. 18:141-148.
7. Laterra, J., R. Ansbacher, and L. A. Culp. 1980. Glycosaminoglycans that bind cold-insoluble globulin in cell-substratum adhesion sites of murine fibroblasts. *Proc. Natl. Acad. Sci. USA*. 77:6662-6666.
8. Stamatoglou, S. C., and J. M. Keller. 1982. Interactions of cellular glycosaminoglycans with plasma fibronectin and collagen. *Biochim. Biophys. Acta*. 719:90-97.
9. Fransson, L.-Å., I. Sjöberg, and B. Havsmark. 1980. Structural studies on heparan sulfates. Characterization of oligosaccharides obtained by periodate oxidation and alkaline elimination. *Eur. J. Biochem.* 106:59-69.
10. Rodén, L., J. R. Baker, J. A. Cifonelli, and M. B. Mathews. 1972. Isolation and characterization of connective tissue polysaccharides. *Methods Enzymol.* 28:73-140.
11. Todaro, G. J. 1969. Transformation assay using cell line 3T3. In *Fundamental Techniques in Virology*. K. Habel and N. P. Salzman, editors. Academic Press, New York. 220-228.
12. Underhill, C. B., and J. M. Keller. 1975. A transformation-dependent difference in the heparan sulfate associated with the cell surface. *Biochem. Biophys. Res. Commun.* 63:448-454.
13. Underhill, C. B., and J. M. Keller. 1977. Heparan sulfates of mouse cells. Analysis of parent and transformed 3T3 cell lines. *J. Cell. Physiol.* 90:53-60.
14. Johnston, L. S., K. L. Keller, and J. M. Keller. 1979. The heparan sulfates of Swiss mouse 3T3 cells. The effect of transformation. *Biochim. Biophys. Acta*. 583:81-94.
15. Keller, K. L., J. M. Keller, and J. N. Moy. 1980. Heparan sulfates from Swiss mouse 3T3 and SV3T3 cells: 0-sulfate difference. *Biochemistry*. 19:2529-2536.
16. Keller, J. M., K. L. Keller, and S. C. Stamatoglou. 1982. Characterization of cell surface heparan sulfates produced by adhesion deficient cell lines. In *The Extracellular Matrix*. S. P. Hawkes and J. L. Wang, editors. Academic Press, New York. 121-125.
17. Ruoslahti, E., E. G. Hayman, M. Pierschbacher, and E. Engvall. 1982. Fibronectin: purification, immunochemical properties, and biological activities. *Methods Enzymol.* 82:803-831.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
19. Grinnell, F., and M. H. Bennett. 1981. Fibroblast adhesion on collagen substrata in the presence and absence of plasma fibronectin. *J. Cell Sci.* 48:19-39.
20. Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4:330-334.
21. Rubin, K., Å. Oldberg, M. Höök, and B. Öbrink. 1978. Adhesion of rat hepatocytes to collagen. *Exp. Cell Res.* 117:165-177.
22. Rubin, K., M. Höök, B. Öbrink, and R. Timpl. 1981. Substrate adhesion of rat hepatocytes: mechanism of attachment to collagen substrates. *Cell*. 24:463-470.
23. Culp, L. A. 1974. Substrate-attached glycoproteins mediating adhesion of normal and virus-transformed mouse fibroblasts. *J. Cell Biol.* 63:71-83.
24. Schubert, D., and M. LaCorbiere. 1980. A role of secreted glycosaminoglycans in cell-substratum adhesion. *J. Biol. Chem.* 255:11564-11569.
25. Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1981. Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell*. 26:254-267.
26. Laterra, J., J. E. Silbert, and L. A. Culp. 1983. Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices, including fibronectin. *J. Cell Biol.* 96:112-123.
27. Kleinman, H. K., A. T. Hewitt, J. C. Murray, L. A. Liotta, S. I. Rennard, J. P. Pennypacker, E. B. McGoodwin, G. R. Martin, and P. H. Fishman. 1979. Cellular and metabolic specificity in the interaction of adhesion proteins with collagen and with cells. *J. Supramol. Struct.* 11:69-78.
28. Hughes, R. C., T. D. Butters, and J. D. Aplin. 1981. Cell surface molecules involved in fibronectin-mediated adhesion. A study using specific antisera. *Eur. J. Cell Biol.* 26:198-207.
29. Knudsen, K. A., P. E. Rao, C. H. Damsky, and C. A. Buck. 1981. Membrane glycoproteins involved in cell-substratum adhesion. *Proc. Natl. Acad. Sci. USA*. 78:6071-6075.
30. Keller, K. L., C. B. Underhill, and J. M. Keller. 1978. Multiple types of cell surface heparan sulfate are produced by primary cultures of embryonic mouse cells. *Biochim. Biophys. Acta*. 540:431-442.
31. Winterbourne, D. J., and P. T. Mora. 1981. Cells selected for high tumorigenicity or transformed by simian virus 40 synthesize heparan sulfate with reduced degree of sulfation. *J. Biol. Chem.* 256:4310-4320.
32. Lindahl, U., and M. Höök. 1978. Glycosaminoglycans and their binding to biological macromolecules. *Annu. Rev. Biochem.* 47:385-417.
33. Hedman, K., S. Johansson, T. Vartio, L. Kjellén, A. Vaheri, and M. Höök. 1982. Structure of the pericellular matrix: association of heparan and chondroitin sulfates with fibronectin-procollagen fibers. *Cell*. 28:663-671.