Fluorescent Morphological Probe for Hyaluronate

CHERYL B. KNUDSON and BRYAN P. TOOLE
Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111

ABSTRACT Hyaluronate levels change dramatically during morphogenesis of various tissues and organs. Morphological detection of the exact temporal and spatial distribution patterns of hyaluronate may help to elucidate its role in morphogenesis. Since no specific direct method for visualizing hyaluronate with the light or electron microscope is currently available, we have developed a morphological probe by exploiting the high-affinity interaction of cartilage proteoglycan with hyaluronate. The core protein of this proteoglycan consists of a region that binds specifically to hyaluronate with a high association constant, and a region to which the majority of sulfated polysaccharide chains are covalently attached. The polysaccharide chains were removed by treatment with chondroitinase ABC, and the core protein, labeled with rhodamine, was used as the probe. This fluorescent probe binds reversibly and specifically to [^{3}H]hyaluronate in a binding assay using ammonium sulfate precipitation of the core protein. The probe has been used to visualize the cell surface hyaluronate of rat fibrosarcoma cells, 3T3 cells, and SV-40 transformed 3T3 cells, three cell types with significantly different amounts of cell surface-associated hyaluronate.

Morphological localization of the spatial pattern of extracellular matrix molecules is necessary to attain a better understanding of their function in developing, remodeling, diseased and normal adult tissues. Antibodies to proteins of the extracellular matrix, such as fibronectin (1, 2), laminin (3–5), and the various collagen types (6–9), have been used extensively to map their location in vivo and to facilitate the development and testing of models for their interactions with other components of the matrix as well as with cells. Many of the proteoglycans present in the extracellular matrix have also been localized by the use of antibodies to their specific core proteins (10, 11). However hyaluronate, a glycosaminoglycan (GAG)[1] which is commonly present in tissues undergoing morphogenesis or remodeling (reviewed in references 12 and 13), probably exists in these tissues as a free GAG polymer to which antibodies have not yet been successfully raised.

To date, no specific direct stain has been developed to visualize hyaluronate in the light or electron microscope. Previous attempts to determine the spatial distribution of hyaluronate have relied upon differences in staining with Alcian blue (14, 15) or [^{3}H]glucosamine autoradiographic grains (16) before and after treatment with hyaluronidases. These methods are most successful when applied to a matrix composed mainly of hyaluronate, since in more complex matrices other GAGs react with Alcian blue or incorporate [^{3}H]glucosamine, thus increasing the background staining. Localizing hyaluronate in fetal or adult tissue that contained several GAG types would be facilitated by use of a more specific stain for hyaluronate.

During tissue fixation, hyaluronate and other GAGs are often extracted, which distorts spatial relationships in the tissue. In recent years, improved fixation techniques, most commonly using tannic acid or cetylpyridinium chloride, reduce the extraction of GAG. However, use of the heavy metal mordant tannic acid in fixatives to localize hyaluronate is complicated by the problem of nonspecificity of its interactions with mucins and other complex carbohydrates (17). Cetylpyridinium chloride used in fixation procedures also is not specific for hyaluronate and will precipitate all GAGs. The precipitates that form within tissues often disrupt cellular integrity and alter the organization of matrix components (18, 19).

In this paper, we describe the development of a fluorescent probe for hyaluronate that exploits the interaction of cartilage proteoglycan with hyaluronate. The core protein of this proteoglycan consists of a region that binds specifically to hyaluronate with a high association constant (20), and a region to which the majority of chondroitin sulfate chains are covalently attached (21). The polysaccharide chains were removed

[1] *Abbreviations used in this paper*: GAG, glycosaminoglycan; RFS, rat fibrosarcoma; SV-3T3, simian virus-40 transformed 3T3.

by treatment with chondroitinase ABC, and the core protein, labeled with rhodamine, was used as the probe. In an alternative probe preparation, the hyaluronate-binding region of the core protein was used. The fluorescent probes interact directly with hyaluronate and are applicable to use with frozen tissue sections, thus eliminating GAG extraction and distortion due to fixation. In this communication, the fluorescent probe has been utilized to visualize the cell surface–associated hyaluronate of three cell types that have been found previously to have distinct differences in cell surface hyaluronate (22): rat fibrosarcoma (RFS) cells, 3T3 cells, and simian virus-40 transformed 3T3 (SV-3T3) cells. Also, the specificity of the probe was analyzed with a specific hyaluronate-binding assay, and by the removal or inhibition of staining by competing agents or enzyme treatment.

MATERIALS AND METHODS

Extraction and Purification of Proteoglycan Monomer: Calf scapular cartilage was ground to a powder in a Wiley mill under liquid nitrogen. The powder was extracted with 9 vol 4.0 M guanidine hydrochloride (practical grade, Sigma Chemical Co., St. Louis, MO), 0.05 M sodium acetate (Sigma Chemical Co.), pH 5.8, which contained the following protease inhibitors: 10 mM ethylenediaminetetraacetic acid disodium salt (Sigma Chemical Co.), 100 mM 6-amino-hexanoic acid (Sigma Chemical Co.), 5 mM benzamidine hydrochloride (Kodak Laboratory, Eastman Kodak Co., Rochester, NY), 2 mM phenylmethylsulfonyl fluoride (Calbiochem-Behring Corp., La Jolla, CA). This solution will be referred to as 4 M GuHCl buffer with protease inhibitors. The cartilage extract was stirred for 90 h at 4°C, centrifuged for 45 min at 13,000 *g* (10,500 rpm in a Sorvall SS-34 rotor, Beckman Instruments, Inc., Palo Alto, CA), and the supernatant was dialyzed against several changes of water for 24 h at 4°C. The material was centrifuged again at 13,000 *g* for 30 min and the supernatant, which contained proteoglycan aggregate, was lyophilized.

To separate proteoglycan monomer from hyaluronate and link protein, a dissociative CsCl gradient was performed according to Hascall and Heinegard (23). 300 mg of proteoglycan aggregate was dissolved in 150 ml of 4 M GuHCl buffer with protease inhibitors plus 3 M CsCl (Accurate Chemical & Scientific Corp., Westbury, NY). The pH was maintained at 5.8. The final density was 1.5 g/ml. Density gradient centrifugation was carried out for 65 h at 4°C at 128,000 *g* (40,000 rpm in a 70 Ti rotor) in a preparative ultracentrifuge (Beckman Instruments, Inc.). The gradient was divided into three equal fractions; the bottom fraction, which contained proteoglycan monomer, was dialyzed against water and lyophilized.

Preparation of Rhodamine-conjugated Core Protein: To form aggregates, 2 mg of hyaluronate (Grade III, Sigma Chemical Co.) was added to 35 mg of proteoglycan monomer dissolved in 3 ml of 0.01 M phosphate buffer, pH 9.3, and the mixture was stirred for 2 h on ice. Rhodamine conjugation was done after a modification of the method reported by Fujiwara and Pollard (24). A solution of 1.5 mg rhodamine-B isothiocyanate (Grade II, Sigma Chemical Co.) dissolved in 0.5 ml of 0.01 M phosphate buffer, pH 9.3 was mixed with the solution of aggregate and stirred on ice for 4 h while the pH was maintained at 9.3. The sample was allowed to stand for 15 h at 4°C, and was then dialyzed against several changes of 0.1 M Tris, 0.1 M sodium acetate buffer, pH 7.4, for 30 h at 4°C, to remove and inactivate the free rhodamine isothiocyanate and to achieve the proper buffering conditions for the following enzyme digestion. 100 μl of a solution of 10 U/ml each of chondroitinase AC (Sigma Chemical Co.) and chondroitinase ABC (Sigma Chemical Co.) in 0.1 M Tris, 0.1 M sodium acetate buffer, pH 7.4 was added and the sample digested for 18 h at 37°C. It was then dialyzed against 4 M GuHCl buffer with protease inhibitors at 4°C for 24 h. The mixture was passed through a Sephacryl S-200 column which had been preequilibrated with the 4 M GuHCl buffer with protease inhibitors, to separate the rhodamine-conjugated core protein from the hyaluronate and chondroitin sulfate fragments. The rhodamine-conjugated core protein eluted in the V_0 fraction, whereas the fragments from the chondroitinase digestion eluted at the total volume. The dye/protein ratio for the rhodamine-conjugated core protein was 1.73.

Preparation of Rhodamine-conjugated Hyaluronate-binding Fragment: After the rhodamine conjugation and dialysis described in the previous section, a limited trypsin (0.0275 mg/ml, Type II, Sigma Chemical Co.) digestion was carried out for 2 h at 37°C, and the reaction was terminated by the addition of soybean trypsin inhibitor (0.26 mg/ml, Type I-S, Sigma Chemical Co.). The sample was dialyzed against a 4 M GuHCl buffer with protease inhibitors at 4°C for 24 h, and then passed through a Sephacryl S-200 column in the same buffer to separate hyaluronate and the chondroitin sulfate–rich region of the proteoglycan monomer from the hyaluronate-binding region of the proteoglycan monomer. These three components eluted at $K_{av} = 0$, $K_{av} = 0.07$, and $K_{av} = 0.64$, respectively. The dye/protein ratio for the rhodamine-conjugated hyaluronate-binding fragment was 0.64.

Hyaluronate Binding Assay: This assay, a modification of that reported by Underhill et al. (25), is based on the reversible binding of the rhodamine-conjugated core protein to [^{3}H]hyaluronate and uses ammonium surface precipitation of the fluorescent probe. The complex of [^{3}H]hyaluronate with the fluorescent probe precipitates, whereas free [^{3}H]hyaluronate remains in the supernatant. The rhodamine-conjugated core protein (protein content determined by Lowry et al. [26]) was mixed with [^{3}H]hyaluronate in 1.5-ml microcentrifuge tubes (Eppendorf) and the final volume adjusted to 0.5 ml with Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS). The mixture contained varying amounts of the fluorescent probe (2.5–60 μg), 3 μg [^{3}H]-hyaluronate (prepared according to Underhill and Toole, reference [27]), 0.5 M NaCl, 0.02 M Tris, pH 8.0. The background was determined by including a large excess of high molecular weight unlabeled hyaluronate (500 μg/ml, Grade I, Sigma Chemical Co.). Samples were shaken at 25°C for 3 h. Casein (1 mg, Sigma Chemical Co.) was added as a protein carrier, and the protein-hyaluronate complex precipitated with an equal volume of saturated ammonium sulfate. After mixing, the samples were centrifuged for 10 min at 12,000 *g* (Microfuge B, Beckman Instruments, Inc.). The supernatants were aspirated and the pellets rinsed three times with 50% saturated $(NH_4)_2SO_4$. The pellets were solubilized in 0.2 M NaOH, the pH of the resulting solutions neutralized, and the radioactivity determined by scintillation counting.

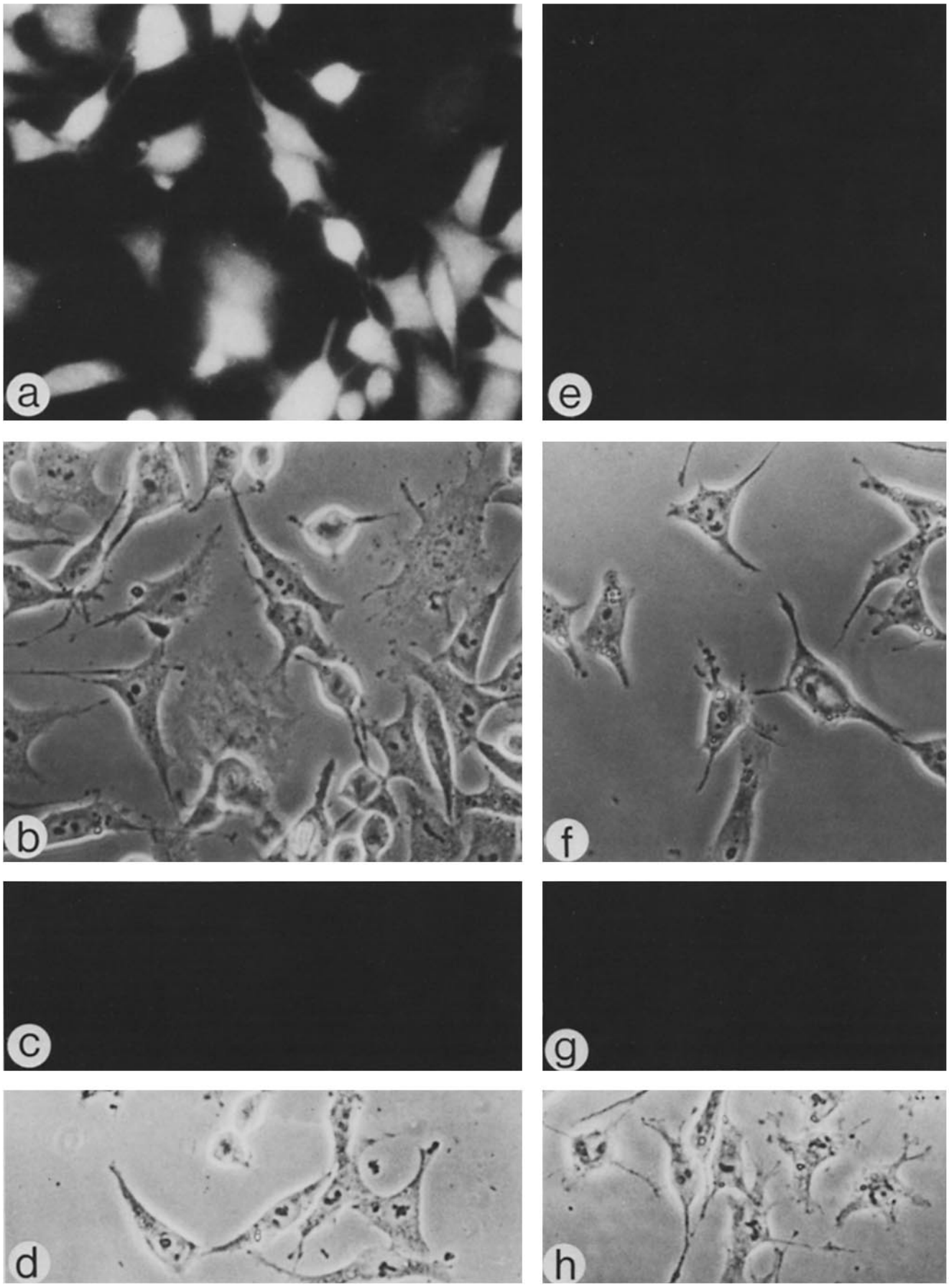
Fixation and Staining of Cells: Most of the studies on the hyaluronate probe presented here were performed with formaldehyde-fixed RFS cells. We also compared the interaction of the probe with formaldehyde-fixed 3T3 and SV-3T3 cells. Cells were grown in Dulbecco's modified Eagle's medium, which contained 4.5 mg/ml glucose, 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY) plus 1% penicillin and streptomycin. Cells were subcultured at low density, 2×10^4 cells/ml, into miniwell culture dishes (Costar Corp., Cambridge, MA) containing glass coverslips (Bellco Glass Inc., Vineland, NJ) and allowed to attach and spread for 1 d. Cells were washed in PBS containing Ca^{++} and Mg^{++} and fixed in 2% formaldehyde (Fisher Scientific Co., Pittsburgh, PA) in PBS, pH 7.4, for 15 min at 25°C, washed and incubated with 180 μl of a solution of the fluorescent probe at various concentrations in PBS. The coverslip was washed and inverted on a glass slide using glycerin with 10% PBS as a support medium. A Zeiss IM 35 inverted microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with phase contrast and bright field optics with Ploem type epiillumination and appropriate filter combinations for visualizing rhodamine was used for observations and photography. All photographs were taken on the same microscope, with the same emission and time of exposure and ASA, developed with the same procedure, and printed to obtain a consistent background.

RESULTS AND DISCUSSION

Staining of RFS Cells with Rhodamine-conjugated Core Protein

RFS cells were used for the initial characterization of the specificity of the fluorescent probe since they have a large amount of cell surface–associated hyaluronate, 0.90 μg/10^6 cells; in comparison, 3T3 cells have 0.33 μg, and SV-3T3 cells

FIGURE 1 Staining of formaldehyde-fixed RFS cells with rhodamine-conjugated core protein. (*a*, *c*, *e*, and *g*) Fluorescence microscopy images; (*b*, *d*, *f*, and *h*) phase contrast images of the same fields as *a*, *c*, *e*, and *g*, respectively. This heterogeneous cell line exhibits two typical morphologies, one very flattened, and the other more round with long filopodia. A few of the very flattened cells exhibited diminished staining, but variability in staining is not common. Cells were incubated with: (*a* and *b*) 3.5 μg of the hyaluronate probe for 3 h; (*c* and *d*) 3.5 μg of the hyaluronate probe for 3 h, then washed and treated with *Streptomyces* hyaluronidase; (*e* and *f*) 3.5 μg of the hyaluronate probe together with exogenous hyaluronate for 3 h; (*g* and *h*) 3.5 μg of the hyaluronate probe together with exogenous, nonconjugated core protein for 3 h. × 275.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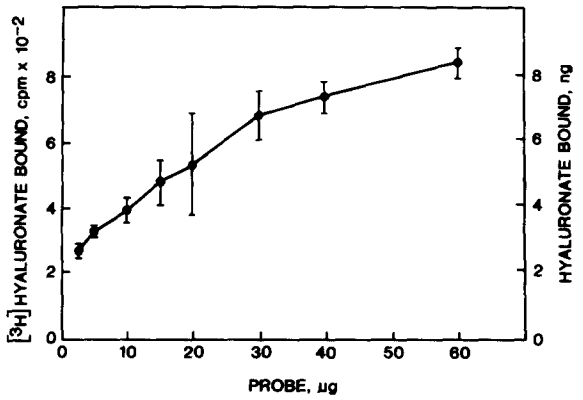

FIGURE 2 Binding of [^{3}H]hyaluronate to rhodamine-conjugated core protein. 3 µg [^{3}H]hyaluronate was added to varying amounts of the fluorescent probe as described in Materials and Methods. All values represent the average of triplicate or quadruplicate determinations minus the nonspecific background. Background was measured by addition of 500 µg unlabeled hyaluronate to the reaction mixture.

have 0.01 µg hyaluronate per 10^6 cells (22). RFS cells were incubated with the rhodamine-conjugated core protein probe using various concentrations of probe and times of incubation. The appearance of visible fluorescence was found to be dependent on both parameters (Table I). On the basis of these

TABLE I

Dependence of Staining on Time and Probe Concentration

	µg Probe					
Time	0.35	0.7	1.75	3.5	5.25	7.0
h						
0.25	−	−	−	−	−	+
0.5	−	−	−	−	−	+
0.75	−	−	−	−	+	+
1	−	−	−	+	+	+
3	+	+	+	+	+	+
5	+	+	+	+	+	+

Formaldehyde-fixed RFS cells were incubated with the above probe concentrations for varying time intervals. −, no detectable fluorescence; +, detectable fluorescence.

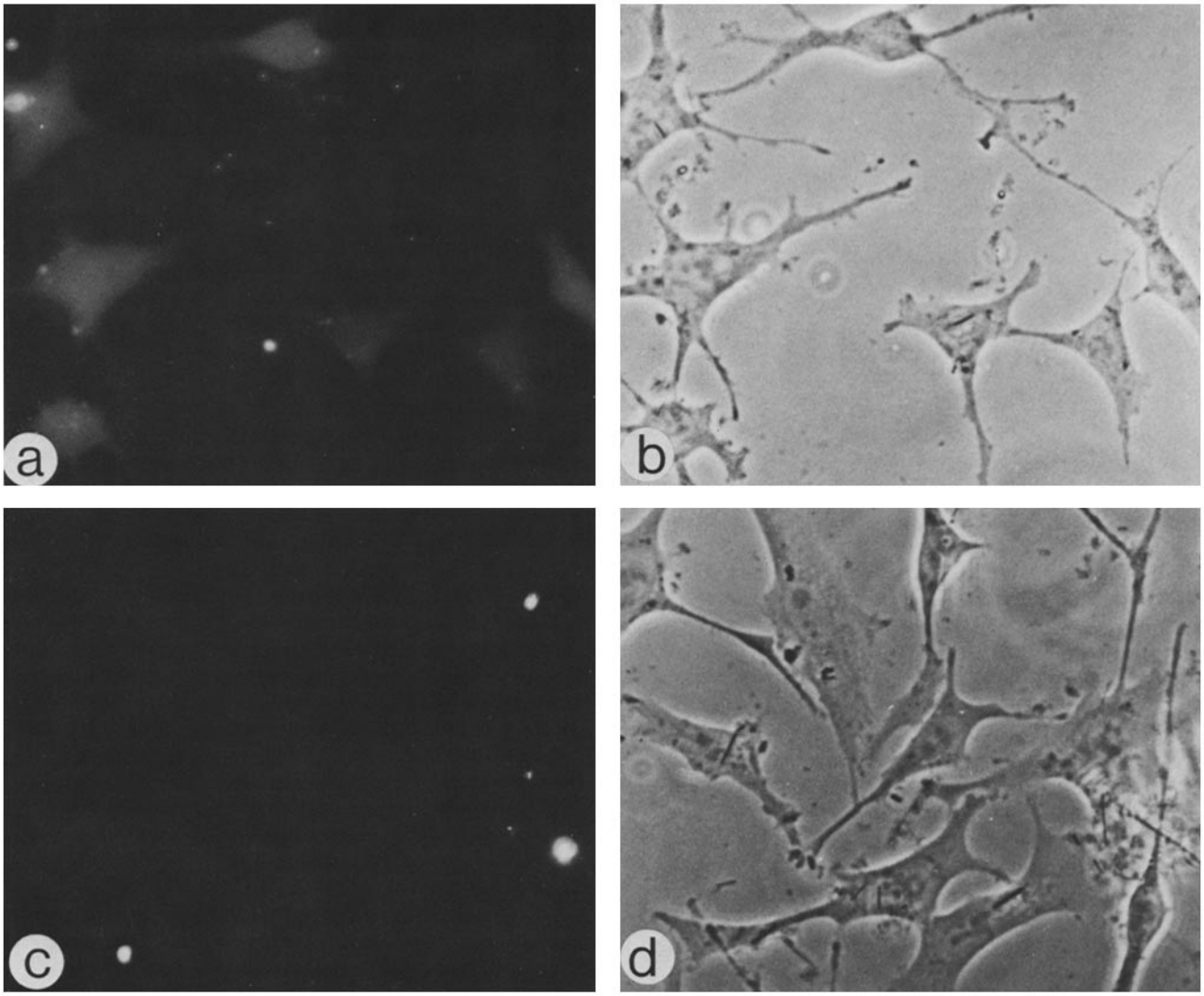

FIGURE 3 Comparison of staining by rhodamine-conjugated hyaluronate-binding and chondroitin sulfate-rich fragments of cartilage proteoglycan. (*a* and *c*) Fluorescence images; (*b* and *d*) phase-contrast images. Formaldehyde-fixed RFS cells were stained with: (*a* and *b*) 7 µg rhodamine-conjugated, hyaluronate-binding fragment for 3 h; or, (c and *d*) 7 µg rhodamine-conjugated chondroitin sulfate-rich fragment for 3 h. × 275.

results, staining in most experiments was performed with 3.5 μg of probe for 3 h at 25°C. Staining under these conditions is shown in Fig. 1 *a*. Staining was inhibited by (*a*) treatment of the cells with enzymes such as *Streptomyces* hyaluronidase, which degrades hyaluronate specifically (28, and Fig. 1 *c*), (*b*) competition with exogenous hyaluronate (Fig. 1 *e*), and (*c*) competition with nonconjugated core protein (Fig. 1 *g*). In each case, titrations were performed to determine the minimum concentrations for inhibition of staining.

TABLE II
Staining of 3T3 and SV-3T3 Cells

	μg Probe					
Time	0.35	0.7	1.75	3.5	5.25	7.0
h						
3T3 Cells						
1	−	−	−	−	−	+
3	−	+	+	+	+	+
SV-3T3 Cells						
1	−	−	−	−	−	+
3	−	−	−	+	+	+

Formaldehyde-fixed 3T3 and SV-3T3 cells were incubated with the above probe concentrations for varying time intervals. −, no observable fluorescence; +, observable fluorescence.

Staining by the fluorescent probe was prevented if the cells were preincubated for 30 min at 37°C with minimum concentrations of 0.01 U/ml *Streptomyces* hyaluronidase (Calbiochem-Behring Corp.), 0.1 ng/ml testicular hyaluronidase (Type I-S, Sigma Chemical Co.), or 0.05 U/ml chondroitinase AC-ABC (Sigma Chemical Co.). If the cells were first incubated with the probe, then treated with *Streptomyces* hyaluronidase, staining was removed. The fluorescent probe might be expected to bind to free link protein in tissues (23), but such staining would not be removed by treatment with hyaluronidases.

Staining was inhibited by competition with nonconjugated core protein; no fluorescence was seen at ratios of nonconjugated-to-conjugated core protein of 32:1 or higher. Similar titrations that used exogenous hyaluronate showed that concentrations of 0.3 mg/ml or greater were inhibitory for staining RFS cells when 3.5 μg of the fluorescent probe was used. However, chondroitin sulfate (Grade III, Sigma Chemical Co., pretreated with *Streptomyces* hyaluronidase to remove any contaminating hyaluronate and then boiled) was not inhibitory at a concentration of 1.5 mg/ml. Inhibition of staining was obtained with nonconjugated core protein or with hyaluronate at the above-mentioned concentrations when the cells were incubated with the probe and competing agent together, and staining was removed when the cells were first incubated with the probe for 3 h, washed, and then incubated

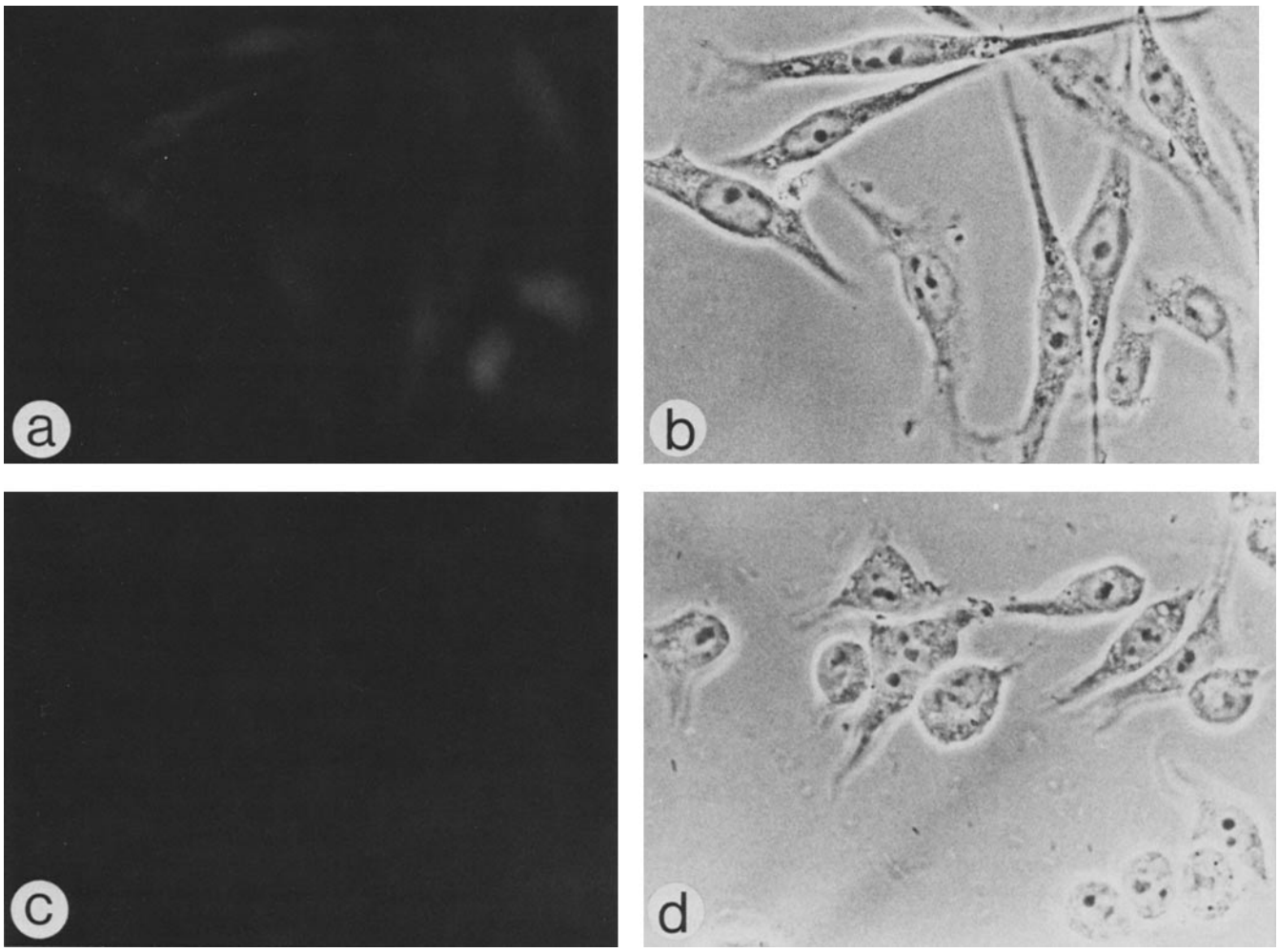

FIGURE 4 Staining of formaldehyde-fixed (*a* and *b*) 3T3 cells and (*c* and *d*) SV-3T3 cells. In both cases, the cells were incubated for 3 h with 3.5 μg rhodamine-conjugated core protein. (a and c) Fluorescence images; (b and d) phase-contrast images. × 275.

with either competing agent.

To confirm the specific binding of the fluorescent probe to hyaluronate, we also tested binding biochemically using the ammonium sulfate precipitation assay. Increasing amounts of [^{3}H]hyaluronate were bound as the protein concentration increased (Fig. 2). Competition of [^{3}H]hyaluronate binding to the probe was obtained with the addition of excess unlabeled hyaluronate. Addition of excess chondroitin sulfate (Grade III, Sigma Chemical Co., pretreated with *Streptomyces* hyaluronidase and then boiled) at equal or greater concentrations (1–2 mg/ml) did not block [^{3}H]hyaluronate binding to the probe.

Staining of RFS Cells with Rhodamine-conjugated Hyaluronate-binding Fragment

In an alternative protocol, the chondroitin sulfate–rich and hyaluronate-binding regions of proteoglycan monomer were separated by gel filtration after limited trypsin digestion of the rhodamine-conjugated monomer. Both fragments were shown to contain conjugated rhodamine molecules. However, only the rhodamine-conjugated hyaluronate-binding region stained the RFS cell surface hyaluronate, as shown in Fig. 3 *a*. The fluorescent signal obtained with this probe was weaker than that with the rhodamine-conjugated core protein (Fig. 1 *a*). This was probably due to the lower dye/protein ratio of the hyaluronate-binding fragment probe. However, an advantage of the rhodamine-conjugated hyaluronate-binding fragment may be its smaller molecular weight which would allow better tissue penetration. Incubation of cells with similar or higher concentrations of the rhodamine-conjugated chondroitin sulfate–rich region did not result in observed fluorescence of the RFS cells (Fig. 3 *c*).

Staining of 3T3 and SV-3T3 Cells with Rhodamine-conjugated Core Protein

The staining of 3T3 cells and SV-3T3 cells was compared using several concentrations of the rhodamine-conjugated core protein probe. SV-3T3 cells, which have 30 times less cell surface–associated hyaluronate than 3T3 cells (22), required higher probe concentrations to observe fluorescence than the 3T3 cells (Table II); thus, qualitatively, the intensity appears to parallel the chemically determined amounts of the cell surface hyaluronate. At those concentrations of probe where both cell types showed staining, the intensity of staining of the 3T3 cells was greater when assessed subjectively. In Fig. 4, 3T3 and SV-3T3 cells were incubated with the rhodamine-conjugated core protein under identical conditions at such a concentration, showing the stronger staining of 3T3 than SV-3T3 cells.

In summary, our preliminary results reported here demonstate the development of a specific fluorescent probe for hyaluronate. The probe, conveniently prepared from cartilage proteoglycan isolated by standard techniques, directly and specifically stains hyaluronate. Future applications of the fluorescent probe may help to elucidate the role of hyaluronate in morphogenesis and tumorigenesis by demonstrating detailed temporal and spatial patterns. The probe has been used successfully with paraffin sections of chick embryo tissues (Toole, B. P., and E. Belsky, unpublished observations) and should be readily applicable to use with frozen tissue sections, thus eliminating GAG extraction and distortion due to fixation. Studies in progress include the use of the fluorescent probe to localize hyaluronate at the connective tissue interface between tumor and neighboring host tissue (29) and in the granule cell layer and other specific regions of normal and mutant mouse brains (30).

The authors thank Charles B. Underhill and Elizabeth Belsky for ideas and assistance in the initial phase of this research, and Bruce E. Batten for advice with the microscopy. A portion of this work was presented at the 22nd Annual Meeting of the American Society for Cell Biology, Baltimore, Maryland, 1982.

The work was supported by a Postdoctoral Fellowship from Muscular Dystrophy Association, and an Individual National Research Service Award GM09235 (to Dr. Knudson), and grant DE05838 from the National Institutes of Health (to Dr. Toole).

Received for publication 21 November 1984, and in revised form 8 January 1985

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