The Hyaluronate Receptor Is Associated with Actin Filaments

Brian E. Lacy and Charles B. Underhill
Department of Anatomy and Cell Biology, Georgetown University, Schools of Medicine and Dentistry, Washington DC 20007

Abstract. The cell-surface receptor for hyaluronate is an integral membrane glycoprotein of Mr 85,000 (Underhill, C. B., A. L. Thurn, and B. E. Lacy, 1985, J. Biol. Chem., 260:8128-8133) that is thought to mediate many of the effects that hyaluronate has on cell behavior, such as migration, angiogenesis, and phagocytosis. To determine if the receptor is associated with the underlying cytoskeleton, Swiss 3T3 cells were extracted with a solution of Triton X-100, which solubilized most of the cellular components, but which left behind an insoluble residue containing the cytoskeleton. This detergent-insoluble residue was found to contain the bulk of the hyaluronate-binding activity, suggesting that the receptor might indeed be associated with the cytoskeleton. To further define the cytoskeletal element with which the receptor interacts, 3T3 cells were extracted with Triton X-100 under a variety of different ionic conditions. In each case, the amount of hyaluronate-binding activity in the detergent-insoluble residue was related to the amount of actin present, but not to either tubulin or vimentin. In addition, the recovery of hyaluronate-binding activity was dramatically enhanced (to 100% in most cases) if the cells were extracted in the presence of phalloidin, a drug that stabilizes actin filaments. However, the recovery of binding activity was dramatically decreased when whole cells were treated with cytochalasin B before extraction, and when extracted cells were treated with DNase I, which promotes the depolymerization of actin filaments. In addition, the recovery of hyaluronate-binding activity was dramatically enhanced (to 100% in most cases) if the cells were extracted in the presence of phalloidin, a drug that stabilizes actin filaments. However, the recovery of binding activity was dramatically decreased when whole cells were treated with cytochalasin B before extraction, and when extracted cells were treated with DNase I, which promotes the depolymerization of actin filaments. In addition, preincubating an extract of SV-40-transformed Swiss 3T3 cell membranes with DNase I caused a change in the elution profile of the receptor as judged by molecular-sieve chromatography. Presumably this decrease in the size of the receptor is due to the loss of associated actin filaments. The results of these experiments strongly suggest that the receptor for hyaluronate is associated either directly or indirectly with cytosolic actin filaments.

ONE of the principal components of the extracellular matrix is hyaluronate, a high molecular mass glycosaminoglycan known to play a vital role in early embryological development (34). The surfaces of most cells contain a receptor for hyaluronate, which we have recently characterized as an integral membrane glycoprotein of Mr 85,000 (39). This receptor may mediate many of the effects that hyaluronate has on cell behavior, including cell-cell adhesion (20, 26, 36), modulation of angiogenesis (11, 40), and stimulation of locomotion and phagocytosis in leukocytes (14, 15).

At present, there is no model to explain how interactions between extracellular hyaluronate and the cell-surface receptor can regulate intrinsic cellular functions such as cell migration and phagocytosis. One obvious possibility is that the hyaluronate receptor may be associated with the cytoskeleton and thereby influence its organization. Indeed, receptors for other components of the extracellular matrix are believed to be associated with the cytoskeleton. For example, the receptor for laminin, an Mr 67,000 glycoprotein (27), has been shown to interact with actin filaments (7). In addition, a number of studies have provided strong evidence that the fibronectin receptor (cell substratum attachment antigen or integrin) is involved in a transmembrane linkage connecting extracellular fibronectin fibrils and intracellular actin filaments (9, 10, 30). Thus, it seemed reasonable to suspect that the receptor for hyaluronate may also be associated with the cytoskeleton.

To test this possibility, we took advantage of the fact that when cultured mammalian cells are extracted with nonionic detergents, all of the soluble proteins, lipids, and most organelles are released into solution, while the nucleus, cytoskeleton, and cytoskeleton-associated proteins are retained in an insoluble residue (1). Under such conditions, we have found that the hyaluronate receptor is preferentially retained in this detergent-insoluble fraction. Furthermore, by manipulating the extraction conditions, the retention of the receptor was directly related to the amount of filamentous actin present in the residue. These results strongly suggest that the receptor is attached either directly or indirectly to cytosolic actin filaments. This in turn indicates a possible mechanism by which extracellular hyaluronate can influence the organization of the cytoskeleton and thereby regulate cell behavior.

Materials and Methods

Cells and Culture Conditions

Swiss 3T3 cells were used in most of the experiments since the cytoskeletal
matrix (cell ghost) of 3T3 cells remains attached to the substratum after detergent extraction. SV-40-transformed Swiss 3T3 (SV-3T3) cells were used in experiments that required large amounts of the receptor. The cells were grown on 100-mm plastic tissue culture dishes (Corning Science Products, Corning, NY) in DMEM supplemented with 10% Nu-Serum (Collaborative Research, Inc., Bedford, MA), and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY). The cells were grown in an atmosphere of 5% CO2/95% air, and were regularly subcultured before reaching confluency.

**Extraction Conditions**

Equal numbers of 3T3 cells were subcultured into a series of tissue culture dishes and allowed to grow until nearly confluent. In most experiments, three individual plates were used for each extraction condition. The medium was removed from the plates, and the cells were washed with a solution containing 1 mM Ca++, 1 mM Mg++, 150 mM NaCl, 15 mM Tris, pH 7.5 (Ca++-Mg++ buffer). The cultures were then placed in an ice-water bath, and extracted with 5 ml per plate of one of the different buffers described below, each of which contained 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, MO). In some experiments, phalloidin (Sigma Chemical Co.) was added to the extraction buffers. For this, a stock solution of 1 mg/ml phalloidin in dimethylsulfoxide was diluted to a final concentration of 10 μg/ml in the extraction buffer. After a 20-min incubation period, the detergent solutions were decanted, and the intact cytoskeleton remaining on the plates was washed twice with the same buffer solution, except that neither detergent nor phalloidin were present. The remaining cytoskeletal matrix was removed from the culture dishes by the addition of 1 ml per plate of 0.1% Na deoxycholate (DOC), 0.5 M NaCl, 0.02 M Tris, pH 8.0 (DOC buffer), which is known to solubilize the hyaluronate receptor (38). Each solution was briefly homogenized for 10 s (Polytron PT20/35; Brinkmann Instruments Co., Westbury, NY) at a setting of 3-4, and then analyzed for various components as described below. In each case, the results from the extracted cultures were compared with control cultures that had been incubated under similar conditions with the Ca++-Mg++ buffer lacking Triton X-100 (i.e., intact cells).

The different buffers used in the extraction experiments consisted of the following: 100 mM NaF, 50 mM KCl, 2 mM Mg++, 20 mM Pipes, pH 6.8 (NaF buffer) (8); 4 mM Mg++, 1 mM EGTA, 150 mM NaCl, 15 mM Tris, pH 7.5 (high Mg++ buffer) (32, 35); 1 mM Ca++, 1 mM Mg++, 150 mM NaCl, 15 mM Tris, pH 7.5 (Ca++-Mg++ buffer) (32, 35); 1 mM Ca++, 1 mM Mg++, 150 mM NaCl, 15 mM Tris, pH 7.5 (Ca++-Mg++ buffer) (32); 600 mM KCl, 4 mM Mg++, 10 mM ATP, 40 mM imidazole, 1 mM EGTA, 1 mM L-cysteine, pH 7.5 (KCl buffer) (6). Note that all buffers, except the DOC buffer, were chilled to 4°C before use.

**Effects of Cytochalasin B**

Cytochalasin B (Sigma Chemical Co.) was dissolved in DMSO (1 mg/ml) and added directly to the medium of three semiconfluent cultures of 3T3 cells to a final concentration of 10 μg/ml. An equivalent amount of DMSO was added to the medium of control cultures. After incubating the cells at 37°C for 3 h, the medium was removed and the cell layers were washed with high Mg++ buffer. Next, a solution of 0.5% Triton X-100 and 1 mM PMSF in high Mg++ buffer (5 ml per plate) was added to the cultures at room temperature, and the cell layers were suspended in this buffer with the aid of a rubber policeman. After centrifugation (17,000 g, 15 min), the pellets were dissolved in DOC buffer, briefly homogenized (Polytron; Brinkmann Instruments Co.), and analyzed for H-hyaluronate-binding activity.

**Effects of DNase I**

To examine the effects of DNase I on the retention of the receptor, nearly confluent cultures of 3T3 cells were extracted on ice with 0.5% Triton X-100 in the high Mg++ buffer as described above. In some cases, phalloidin (10 μg/ml) was added to the extraction buffer. After the final wash in detergent-free, phalloidin-free, high Mg++ buffer, the cultures were incubated in the presence and absence of 1 mg/ml DNase I (type II; Sigma Chemical Co.) dissolved in a buffer that consisted of 4 mM Mg++, 50 mM Tris, 1 mM PMSF, pH 7.2 (2.5 ml per plate) (31). After a 45-min incubation at 25°C, the material associated with each culture plate was collected with a rubber policeman, centrifuged (17,000 g, 15 min), and the pellets solubilized in DOC buffer. The samples were then assayed for both protein content and hyaluronate-binding activity.

The effects of DNase I on the receptor were examined further using column chromatography. In this experiment, a membra-rich fraction was prepared from SV-3T3 cells using differential centrifugation as described previously (38). One-half of this preparation was dissolved in 3 ml of 0.3 mg/ml DOC, 0.02 M Tris, pH 8.0 and applied directly to a 1.5 x 120 cm column of Sepharose CL-6B. The second half of the membrane preparation was dissolved in the same buffer and incubated with DNase I (1.7 mg/ml final concentration) at 25°C for 2 h before application to the column of Sepharose CL-6B. To inactivate any residual protease activity, the preparation of DNase I was preincubated for 1 h with 1 mM PMSF in 1 mM MgCl2, 0.02 M Tris, pH 7.5, before mixing with the membrane extract. In each case, the columns were eluted with 0.1% DOC, 0.02 M Tris, pH 8.0, and 2-ml fractions were collected and assayed for both protein and [3H]-hyaluronate-binding activity (see below). The total volume was determined by including a small amount of [3H]-acetate with the sample.

To determine that the effects of DNase I were not due to the presence of contaminating proteases, the DNase I was assayed for proteolytic activity by a modification of the method of Tomarelli et al. (33). The DNase I (1.7 mg/ml final concentration) was incubated as before for 1 h with 1 mM PMSF in 1 mM MgCl2, 0.02 M Tris, pH 7.5. 1 vol of the DNase I solution was then mixed with 1 vol of azoalbunin (Sigma Chemical Co.), which had been dissolved in 0.02 M Tris, pH 8.0, to a final concentration of 2.5 mg/ml. This solution was incubated for 2 h at 25°C. Next, 4 vol of 5% TCA was added, and the sample was centrifuged for 5 min at 9,000 g in a microfuge (Beckman Instruments, Inc., Palo Alto, CA). The supernatant was removed, mixed with an equal volume of 0.5 N NaOH, and the DNA was determined. The DNase I samples were compared with a series of trypsin standards that had been treated in an identical manner; the standards were found to be sensitive to proteolytic activity of <1 μg trypsin/mg. Under these conditions, no proteolytic activity of the sample found in the DNase I preparation after pretreatment with 1 mM PMSF.

**Biochemical Assays**

The protein content of the samples was determined using the standard Lowry assay (21). To compensate for the effects of the DOC buffer, the protein standards were also dissolved in this buffer.

The hyaluronate-binding activity of the extracts was determined as follows. Aliquots (460 μl) of the samples dissolved in DOC buffer were added to 1.5-ml centrifuge tubes (Eppendorf; Brinkmann Instruments Co.) and mixed with 2.0 μg of [3H]-hyaluronate (20 μl of a 100 μg/ml solution; 2.6 x 106 cpm/μg, which was prepared as previously described (37, 38). After shaking the samples for 15 min at 25°C, 200 μl of saturated (NH4)2SO4 was added, and the samples were vigorously shaken to ensure proper mixing. Next, 50 μl of skim milk was added as a protein carrier, the samples were briefly shaken, and then centrifuged for 5 min at 9,000 g (Microfuge B; Beckman Instruments, Inc.). The tubes containing the precipitates were carefully washed three times with 50% saturated (NH4)2SO4. Each precipitate was dissolved in 1 ml of water and then processed for scintillation counting. The background level of binding was determined by including 200 μg (20 μl of a 10 mg/ml solution) of nonlabeled hyaluronate (type I; Sigma Chemical Co.) in the assay. The results are expressed in terms of specific binding in which the background has been subtracted. Each sample was assayed in duplicate and in all cases the values differed by no >10% from the mean.

To determine if actin itself could bind hyaluronate, a preparation of actin (both the F and G forms) purified from rabbit muscle (25) was dissolved in DOC buffer (1.0 mg/ml) and then 460 μl of this solution was tested directly in the [3H]-hyaluronate-binding assay. A commercial form of actin (Sigma Chemical Co.) was also tested in a similar manner.

The actin content of the samples was determined using the DNase I inhibition assay developed by Bildstod and co-workers (3, 4). The cells were extracted with Triton X-100 and washed with the corresponding buffer as described above. The detergent-insoluble residue was then suspended in distilled water with the aid of a rubber policeman and homogenized briefly with a polytron. Aliquots from each sample were diluted with an equal volume of depolymerization buffer (1.5 M guanidine HCl, 1 M Na acetate, 1 mM
CaCl₂, 1 mM ATP, 20 mM Tris, pH 7.5) and placed in an ice-water bath for 10-30 min. The samples (10 µl) were then quickly mixed with an equal volume of a 0.1 mg/ml solution of DNase I dissolved in 1.0 mM CaCl₂, 0.01 mM PMSEF, 50 mM Tris, pH 7.5. This mixture was added 1 ml of DNA substrate (40 µg/ml DNA dissolved in 0.1 M Tris, 4 mM MgSO₄, 1.8 mM CaCl₂, pH 7.5). The change in OD₅₀₀ was monitored for a period of several minutes. The amount of actin in each sample can then be determined by comparing the degree of inhibition of DNase I activity with that obtained with known amounts (0-60 µg/ml) of purified actin. It should be noted that this assay cannot be used to determine the amount of actin in phalloidin-treated samples since phalloidin interferes with the depolymerizing effects of DNase I on actin (28).

**SDS-PAGE**

The cells were extracted under various conditions, and the insoluble residues were digested directly in Laemmli sample buffer containing beta-mercaptoethanol (18). The samples were placed in a boiling water bath for 3 min and then subjected to electrophoresis on a 10% SDS-polyacrylamide gel. To stain with Coomassie Blue, the gel was immersed for 30 min in 0.1% Coomassie Blue in 40% methanol, 10% acetic acid, and then destained in 10% methanol, 75% acetic acid. Actin and vimentin were identified as bands at M, 43,000 and 58,000, respectively (13).

**Isotopic Labeling of Carbohydrates**

To determine the effects of detergent extraction on the cell's carbohydrate content, 3T3 cells were cultured in a glucose-free, DME-10% Nu-Serum medium containing 0.4 µCi/ml of 6-[3H]-glucosamine (ICN Pharmaceuticals, Inc., Irvine, CA). After 18-20 h of incubation, the cells were extracted with the appropriate buffer containing 0.5% Triton X-100 as described above. The matrix was then solubilized in 5 ml per plate of 1% SDS, and the duplicate samples for each condition were kept separate. Next, the samples were dialyzed against three changes of 0.1% SDS, 0.1 M NaCl, 0.02 M Tris, pH 7.5 (SDS buffer). The samples were precipitated by the addition of 4 vol of 1.3% potassium acetate in 95% ethanol, maintained at -20°C for 4 h, and then centrifuged at 900 g in a clinical centrifuge. The precipitate was dissolved in 0.1 M NaCl, 0.02% Na azide, 0.02% Tritos, pH 7.5, and then digested for 24 h at 37°C with 1 mg/ml protease (type VI; Sigma Chemical Co.). After the incubation period, the medium was removed from both groups, and the plates were re-run on the Sephadex column. The amount of hyaluronate was then determined from the fraction of material present in the included volume.

**Hyaluronate Assay**

To determine the amount of endogenous hyaluronate remaining with the cells after treatment with testicular hyaluronidase, 3T3 cells were grown for 313 cells were grown for 18-20 h in medium containing [3H]glucosamine as described above. The culture dishes were then solubilized with 5 ml per plate of 1% SDS, and processed similarly to the [3H]glucosamine-labeled cells described in the section above, except that the samples were eluted from the Sephadex G-100 column with a buffer that lacked SDS (i.e., 0.1 M NaCl, 0.02 M Tris, 0.02% Na azide, pH 7.5). The material in the void volume was then collected and digested with 10 units of Streptomyces hyaluronidase (Miles Laboratories, Inc., Naperville, IL), at pH 6.0 for 18-20 h at 4°C. The resulting digests were lyophilized, dissolved in the same buffer described above, and then rerun on the Sephadex column. The amount of hyaluronate was then determined from the fraction of material present in the included volume.

**Microscopic Techniques**

The 3T3 cells were cultured on glass coverslips under conditions identical to those described above. To determine the presence of cytoskeletal components before detergent extraction, subconfluent 3T3 cells were washed with CaCl₂-free buffer and then simultaneously fixed and permeabilized in a solution of 1.0% paraformaldehyde, 2.5% glutaraldehyde, 0.25% Triton X-100 in PBS, pH 7.4, for 10 min at 25°C. To determine the effects of detergent extraction on actin content, coverslips were extracted for 20 min on ice with the Triton X-100-containing buffers described above. Next, the coverslips were washed in PBS and fixed for 10 min at 25°C with a 4% paraformaldehyde solution in PBS.

To stain for actin filaments, the coverslips were incubated at 37°C for 20 min with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Junction City, OR) diluted 1:100 in PBS. To identify tubulin and vimentin, the slides were incubated with the primary antibodies diluted in PBS for 1 h at 37°C, washed five times with PBS, and then incubated with the secondary antibody diluted in PBS for 1 h at 37°C. The primary antibodies used were rabbit anti-chicken tubulin and goat anti-mouse fibroblast vimentin (both from Miles Laboratories, Inc.). The secondary antisera used included a rhodamine-conjugated goat F(ab')₂ fragment produced against rabbit IgG (H and L chains specific; Accurate Chemical and Scientific Corp., Westbury, NY), and a rhodamine-conjugated rabbit IgG produced against goat IgG (H and L chains specific; Cappel Laboratories, Inc., Cochranville, PA).

Both the primary and the secondary antibodies were used at a dilution of 1:100. After staining, the coverslips were washed extensively with PBS and inverted onto a glass slide in a 1:1 glycerol/PBS solution, and viewed under a Zeiss Photomicroscope III equipped for epifluorescence.

**Results**

**Effects of Detergent Extraction on the Hyaluronate Receptor**

Initial experiments were designed to determine whether the hyaluronate receptor is retained in the detergent-insoluble residue after detergent extraction under normal physiologic conditions of pH and ionic strength. To accomplish this, confluent cultures of 3T3 cells were extracted at 4°C for 20 min with 0.5% Triton X-100 in Ca²⁺-Mg²⁺ buffer (see Fig. 1). The 3T3 cells extracted in this manner remained adherent to both glass coverslips and tissue culture plates, and neither detached nor rounded up. As shown in Fig. 1, detergent extraction of 3T3 cells produced cell ghosts consisting of the nucleus embedded in a meshwork of wispy filaments. The thin, wavy strands of filamentous material primarily represent the collapsed cytoskeleton. Analysis of this insoluble material revealed that it contained only 31% of the cellular protein, but 55% of the H-hyaluronate-binding activity (see Table I, Ca²⁺-Mg²⁺ buffer). These initial results suggested that the receptor is specifically retained in the detergent-insoluble residue.

One possible explanation for the above result is that the buffer solution did not extract cell surface hyaluronate, which in turn prevented the release of the receptor from the matrix. To test this possibility, 3T3 cells were treated with hyaluronidase to remove most of the cell surface-associated hyaluronate, and then were extracted as described above. While this pretreatment with hyaluronidase released 80% of the hyaluronate, it had only a small effect on the retention of the hyaluronate-binding activity (8,076 cpm for the control versus 7,467 cpm for the hyaluronidase-treated sample). These results suggest that cell surface-associated hyaluronate is not responsible for retaining the receptor in the Triton-insoluble residue. Rather, the receptor is most likely associated either directly or indirectly with the cytoskeleton.

The cytoskeleton of 3T3 cells, like that of most other eukaryotic cells, contains three major types of filaments: microtubules, intermediate filaments, and actin filaments. Intermediate filaments are a class of related proteins of which only one, vimentin, is found in 3T3 cells (13). To determine which of the three cytoskeletal components is involved in the retention of the hyaluronate receptor, we used immunocytochemical techniques to examine the recovery of each element after detergent extraction. As shown in Fig. 1, microtu-
Figure 1. Comparison of 3T3 cells before and after detergent extraction. To determine the effects of detergent extraction on cytoskeletal elements, one set of 3T3 cells was extracted for 20 min at 4°C with 0.5% Triton X-100 in Ca++-Mg++ buffer and subsequently fixed with formaldehyde (b, d, f, and h). This was compared with a control set of unextracted cells (a, c, e, and g) that was simultaneously fixed and permeabilized as described in Materials and Methods. (a and b) Phase contrast of control and detergent-extracted cells, respectively. (c and d) Immunofluorescent staining of tubulin in control and detergent-extracted cells, respectively. (e and f) Immunofluorescent staining of vimentin filaments in control and detergent-extracted cells. (g and h) Rhodamine-conjugated phalloidin staining of actin filaments in control and detergent-extracted cells. Bar, 10 μm.
bubbles were no longer present in the cytoskeletal matrix after detergent extraction at 4°C, which would be expected since low temperatures are known to depolymerize microtubules. This result eliminated the possibility that microtubules are involved in the receptor–cytoskeleton interaction. However, the extracted cells stained positively for both vimentin (Fig. 1 f) and actin (Fig. 1 h), and for this reason we investigated these two components further.

Effects of Different Extraction Conditions

The 3T3 cells were extracted with Triton X-100 under a variety of buffer conditions that have been reported to differentially affect the recovery of actin. When examined by phase contrast microscopy, no obvious differences were observed between the cells extracted under the five different buffer conditions (data not shown). However, when these matrices were stained for actin filaments, significant differences were apparent. For example, when cells were extracted with the NaF buffer (see Fig. 2 b), which stabilizes F-actin (8), the pattern of actin filaments was very similar to that of unextracted cells (Fig. 2 a). In both cases, the actin filaments were present in numerous stress fibers, and in a layer immediately beneath the plasma membrane. In addition, other filaments appeared to be approaching and terminating either at or near the plasma membrane, which presumably reflects the presence of actin in the focal contacts and spread margins of the cell. In contrast, cells extracted in the KCl buffer (Fig. 2 c), a condition designed to depolymerize actin filaments (6), had a dramatically altered staining pattern. Stress fibers were no longer apparent, the total amount of actin present appeared to be greatly reduced, and the small amount of actin remaining was chiefly located around the cell periphery. Cells extracted under the other three conditions produced actin staining patterns intermediate to those just described (not shown).

The 3T3 cells extracted under different conditions were also analyzed biochemically (Table I). The actin content of the different cell extracts was determined by measuring the inhibition of DNase I activity (3, 4). The results of this assay supported our earlier observations made by the fluorescent staining of the extracted cells (see Table I). The extracts were also analyzed for both total protein and glycoprotein-associated carbohydrate. This latter value was determined because the hyaluronate receptor has been shown to be a glycoprotein (39), and thus it is appropriate to compare the recovery of the receptor with the recovery of glycoprotein-associated carbohydrate. The comparison between recovery of binding activity and recovery of glycoprotein-associated carbohydrate may in fact be the most valid, since the total protein content of the detergent-insoluble residue includes a large fraction of nonglycosylated proteins present in the nuclei, which is clearly not associated with the cytoskeleton. As shown in Table I, each extraction condition released a greater fraction of glycoprotein-associated carbohydrate as compared with total protein.

It is also apparent from Table I that the recovery of the hyaluronate-binding activity was always greater than that of either total cellular protein or of glycoprotein-associated carbohydrate. Indeed, the rank order of hyaluronate-binding activity paralleled that for the recovery of actin across the different extraction conditions. For example, cells extracted in the NaF buffer retained the maximal amount of actin (61%) as well as the greatest amount of hyaluronate-binding activity (82%). In contrast, cells extracted with the KCl buffer, retained the least amounts of actin (15%), and the smallest amount of hyaluronate-binding activity (23%). The other extraction buffers resulted in the retention of intermediate values of both actin and hyaluronate-binding activity.

One trivial explanation for the results described above is that actin itself is binding the 3H-hyaluronate. To test this possibility, we examined several preparations of F-actin in our binding assay to determine whether actin could bind 3H-hyaluronate. No binding was detected even with relatively high concentrations of the purified actin. This precludes the possibility that a direct interaction occurs between actin and hyaluronate and suggests that the most likely explanation for the results described above is that the receptor is associated in some fashion with actin filaments.

Effects of Phalloidin on the Recovery of the Receptor

The possibility of an association between the hyaluronate receptor and actin filaments was examined further by extracting cells in the presence of phalloidin, a peptide which stabilizes filamentous actin (F-actin) (19). Phalloidin is highly specific in its interaction with actin, and it neither binds to nor stabilizes intermediate filaments. As seen in Table II, the
addition of phalloidin to the extraction buffer produced nearly complete retention of the hyaluronate receptor in most cases, and caused only a slight increase in the protein content of the extracted cells, which is most likely due to an increase in the amount of F-actin retained. In 3T3 cells, actin may constitute 10–15% of the total cellular protein, hence this small increase in the amount of protein retained is not surprising. More significant, however, is the retention of 90% of the binding activity in cells extracted with the KCl buffer containing phalloidin, in contrast to the 23% retention of binding activity when cells were extracted with the KCl buffer in the absence of phalloidin. This result is consistent with other studies which have shown that F-actin, if previously stabilized by phalloidin, can withstand the depolymerizing effects of high ionic strength solutions (19).

To determine the effect of phalloidin on the recovery of actin, each of the cell extracts was analyzed by SDS-PAGE (Fig. 3). This procedure was used because phalloidin interferes with the analysis of actin by the DNase I inhibition assay (28). Although the results obtained by SDS-PAGE are only semiquantitative, it is apparent that for each extraction condition, the presence of phalloidin significantly enhanced the recovery of actin.

Fig. 3 also shows that the recovery of vimentin did not correlate with the recovery of hyaluronate-binding activity. First, for each individual extraction condition, the presence of phalloidin did not substantially alter the recovery of vimentin. Yet, the recovery of both actin and hyaluronate-binding activity were greatly enhanced. And secondly, when the recovery of vimentin was compared between the different extraction conditions, it was clear that it did not correlate with the recovery of hyaluronate-binding activity. This can be readily appreciated by comparing Fig. 3, lane 7 (cells extracted in Ca++-Mg++ free buffer) with lane 10 (cells extracted with phalloidin in Ca++-Mg++ buffer). The sample in Fig. 3, lane 7 clearly contains much more vimentin than that in lane 10, even though it has less 3H-hyaluronate-binding activity associated with it (66 vs. 100%). The results of these experiments indicate that the recovery of the hyaluronate receptor is not related to the recovery of vimentin and suggest that there is not a productive interaction between the two.

Effects of Cytochalasin B on the Recovery of the Receptor

Cytochalasin can inhibit the rate of elongation of actin filaments by binding to the barbed, fast growing end of F-actin, and can also alter the morphology and disrupt the cytoskeletal network of cultured cells (5, 12, 16, 22, 23, 29). To examine the effects of this drug on the recovery of the hyaluronate receptor, nearly confluent cultures of 3T3 cells were treated with cytochalasin B for 3 h. Cytochalasin B treatment resulted in extensive morphological changes in 3T3 cells, including cell retraction and arborization (data not shown). After the incubation period, the cells were extracted with a solution of Triton X-100 and then centrifuged to separate the

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**Table II. The Effects of Phalloidin on the Extraction of Protein and 3H-hyaluronate-binding Activity**

| Extraction condition | Protein  | Hyaluronate-binding activity |
|----------------------|----------|----------------------------|
| NaF buffer           | 42.3 ± 1.4 | 112 ± 1                      |
| High Mg++ buffer     | 40.5 ± 1.5 | 108 ± 8                      |
| Ca++-Mg++ free buffer| 36.5 ± 2.5 | 98 ± 8                       |
| Ca++-Mg++ buffer     | 31.0 ± 1.2 | 99 ± 1                       |
| KCl buffer           | 25.9 ± 2.2 | 90 ± 10                      |

* The 3T3 cells were extracted according to the method outlined in Table I except that the extraction buffer contained 10 μg/ml phalloidin.

* All values are compared with controls that consisted of intact cells that had been incubated in the Ca++-Mg++ buffer (without Triton X-100) for 20 min at 4°C. The values shown are the average and range from two independent observations.
detergent-insoluble fraction from the detergent-soluble fraction. When the precipitate was assayed, it was found to contain 63% (SD = 4.0; n = 3) of the 3H-hyaluronate-binding activity as compared with precipitates from control cells that had not been pretreated with cytochalasin B. These results are consistent with an association between the receptor and actin filaments, which is disrupted by cytochalasin B.

When the detergent-insoluble fractions were analyzed by SDS-PAGE, we found little if any difference in the amount of F-actin retained in cells treated with cytochalasin B, as compared with those treated with DMSO (data not shown). These results are consistent with those of Morris and Tannenbaum (24), who found that cytochalasins do not produce a net depolymerization of actin filaments, even though the concentrations used were sufficient to produce a disruption of the cytoskeletal network, as evidenced by cell retraction and cell arborization. Although the exact mechanism by which cytochalasin B produces a loss of the receptor from the detergent-insoluble fraction is not known, it is quite possible that it inhibits some type of interaction or association between the hyaluronate receptor and F-actin.

Effects of DNase I on the Recovery of the Receptor

Through its ability to bind tightly to globular or free actin (G-actin), DNase I can prevent G-actin from polymerizing into filamentous actin. Additionally, when DNase I binds to G-actin, it can rapidly alter the equilibrium between F- and G-actin, and if present in sufficient quantities, it can cause the depolymerization of filamentous actin (17). To examine the effects of DNase I on the recovery of the receptor, confluent cultures of 3T3 cells were first extracted with Triton X-100, and then the material remaining on the culture dish was further incubated in the presence and absence of DNase I. After this incubation, the material in each dish was suspended in buffer and then centrifuged. Table III shows that cytoskeletal matrices treated with DNase I lost a significant portion of their hyaluronate-binding activity, as compared with matrices incubated with buffer alone (34 vs.

![Figure 3. A Coomassie Blue-stained SDS-polyacrylamide gel (10%) of the detergent-insoluble residue from 3T3 cells after extraction under different conditions. The cells were extracted with Triton X-100 in various buffers both in the presence and absence of phalloidin. The different extraction buffers consisted of the following: lanes 3 and 4, NaF buffer; lanes 5 and 6, high Mg++ buffer; lanes 7 and 8, Ca++-Mg++ free buffer; lanes 9 and 10, Ca++-Mg++ buffer; and lanes 11 and 12, KCl buffer. For each extraction buffer, the first lane of each set consists of cells extracted in the presence of phalloidin, while the second lane consists of cells extracted in the presence of phalloidin. Lane 1 contains molecular mass standards, while lane 2 contains a homogenate of intact (unextracted) 3T3 cells. The bands corresponding to actin (Mr 43,000) and vimentin (Mr 58,000) are indicated on the right side of the figure.](image)

| Group | First buffer | Second buffer | Total protein | Total binding |
|-------|--------------|---------------|---------------|---------------|
| A     | –            | –             | 1.56          | 100           |
| B     | –            | DNase I       | 1.30          | 83            |
| C     | Phalloidin   | –             | 1.58          | 101           |
| D     | Phalloidin   | DNase I       | 1.47          | 94            |

* Nearly confluent cultures of 3T3 cells were extracted with 0.5% Triton X-100 and 1 mM PMSF in the high Mg++ buffer as described in Table I. Groups A and B (three plates each) were incubated in the absence of phalloidin; groups C and D (three plates each) were incubated in the presence of 10 μg/ml phalloidin. After decanting the extraction buffer, and washing the plates twice in the detergent-free high Mg++ buffer, groups A and C were incubated with 2.5 ml per plate of a buffer solution (50 mM Tris, 4 mM Mg++, 1 mM PMSF, pH 7.2). Groups B and D were incubated with 2.5 ml per plate of 1 mg/ml DNase I (type II; Sigma Chemical Co.) dissolved in the same buffer. Incubations were for 45 min at 25°C. Samples were collected with a rubber policeman, centrifuged (17,000 g, 15 min), the supernatants decanted, and the pellets solubilized in DOC buffer. The samples were assayed for both protein content and hyaluronate-binding activity.

+ All values are compared with controls that consisted of cells extracted in the absence of both phalloidin and DNase I (group A). These values are from a single experiment, although they are representative of four independent observations.
As judged by column chromatography
tions of the void protein (e) and 3H-hyaluronate-binding activity (o). The posi-

membrane extract was pretreated with 1.7 mg/ml of DNase I in 1 mM
Mg +§ 1 mM PMSF for 2 h at room temperature, and then ap-
and eluted with 0.1% DOC, 0.02 M Tris, pH 8.0. (B) The mem-

Figure 4. The elution profiles of the hyaluronate receptor from
Sepharose C1-6B, both before (A) and after (B) treatment with
DNase I. Membranes were isolated from SV-3T3 cells and dis-
solved in 0.3% DOC, 0.02 M Tris, pH 8.0. (A) The extract was
applied directly to a 1.5 x 120 cm column of Sepharose C1-6B
and eluted with 0.1% DOC, 0.02 M Tris, pH 8.0. (B) The mem-
brane extract was pretreated with 1.7 mg/ml of DNase I in 1 mM
Mg++, 1 mM PMSF for 2 h at room temperature, and then ap-
plied to the column. 2-ml fractions were collected and assayed for
protein (solid line) and 3H-hyaluronate-binding activity (open line).
The positions of the void (V0) and the total volumes (Vt) are indicated.
Different preparations of 3H-hyaluronate were used to assay bind-
ing activity in A and B, which accounts for the differences in the
total binding activity present. Results similar to those shown in this
figure were obtained in three separate experiments.

Discussion
In this report we have presented several lines of correlative
evidence that suggest that the hyaluronate receptor is either
directly or indirectly associated with cytosolic actin fil-
ments. First, when 3T3 cells were extracted under a variety
of different conditions, the amount of hyaluronate-binding
activity remaining in the insoluble matrix was directly re-
lated to the amount of actin present, but not with either
vimentin or tubulin. Secondly, when the cells were extracted
in the presence of phalloidin, which specifically stabilizes
actin filaments, most if not all of the binding activity was re-
tained in the detergent-insoluble residue. This occurred even
under conditions that in the absence of phalloidin resulted in
the loss of most of the binding activity (e.g., 600 mM KCl).
Thirdly, pretreatment of 3T3 cells with cytochalasin B, which
disrupts the cytoskeleton and inhibits F-actin elongation,
decreased the recovery of the receptor after detergent ex-
traction. Fourthly, when cell extracts were treated with DNase
l, which causes the depolymerization of actin filaments,
most of the binding activity was lost from the detergent-
 insoluble fraction. However, much of this effect could be
blocked if the actin filaments were first stabilized with phal-
loidin. And finally, the size of a complex containing the
receptor was decreased after treatment with DNase I, which
is presumably due to the depolymerization of actin filaments.
While these results are only correlative in nature, they do
strongly suggest that the hyaluronate receptor is associated
either directly or indirectly with actin filaments.

The results described above also suggest that this associa-
tion is of a specific nature, and not due to a simple physical
trapping of the receptor in a meshwork of actin filaments.
While trapping could account for a small fraction of the
receptor being retained in the detergent-insoluble fraction, it
is unlikely that it could account for the nearly quantitative
recovery of the receptor in this fraction after detergent ex-
traction in the presence of phalloidin. This is particularly
ture in view of the fact that most of the receptor is located
on the cell surface where it is improbable that it could be-
come enmeshed in the actin matrix. In addition, the recep-
tor was found to be present in a large complex as judged by
molecular-sieve chromatography in a low ionic strength
buffer. Again, it is unlikely that the receptor could remain
entrapped in a network of actin filaments during the entire
process of chromatography. Thus, the most likely explana-

Effect of DNase I on the Size of the Receptor
As Judged by Column Chromatography
The interaction between the receptor and actin (whether di-
rect or indirect) could account for an observation made in an
earlier study concerning the size of the receptor complex.
We had found that when membranes of SV-3T3 cells were
solubilized with DOC in a high ionic strength buffer (0.1%
DOC, 0.5 M NaCl, 0.02 M Tris, pH 8.0), the receptor eluted
from a column of Sepharose CL-6B with a Kav of 0.46 (39).
However, when the membranes were extracted and chro-
matographed with DOC in a low ionic strength buffer (0.1%
DOC, 0.02 M Tris, pH 8.0), then the receptor eluted predom-
inantly in the void volume (see Fig. 4 A). It is possible that
this difference in the size of the receptor complex was due
to the presence or absence of F-actin. In the low ionic
strength buffer, the F-actin would be stable and hence the
receptor would appear to be very large. However, in a high
ionic strength buffer, F-actin would be depolymerized, and
the receptor would take on a smaller size.

To test this possibility, membranes from SV-3T3 cells were
dissolved in a low ionic strength buffer and then incubated
with a high concentration of DNase I to depolymerize the ac-
tin filaments. When the resulting extract was examined by
molecular-sieve chromatography on Sepharose CL-6B, the
bulk of the receptor activity eluted in the included volume
of the column with a Kav of 0.29 (see Fig. 4 B). Again,
these results support our hypothesis that the receptor is as-
associated with actin filaments, which in turn are sensitive to
depolymerization by DNase I.

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tion is that the receptor is specifically associated with actin filaments either in a direct or indirect fashion.

Further support for this conclusion comes from an immunohistochemical study by Guido Tarone and his associates (32). These investigators have isolated a monoclonal antibody (K-3) to an Mr 85,000 glycoprotein on the surface of baby hamster kidney (BHK) cells which they have termed gp85. In a collaborative study between Dr. Tarone's laboratory and our own, we have found that gp85 is identical to the hyaluronate receptor based upon its cross-reactivity with the K-3 antibody. When BHK cells were stained immunohistochemically with the K-3 monoclonal antibody, the hyaluronate receptor was found to be located in small patches that were closely associated with stress fibers of filamentous actin (32). These findings provide further support for the association between the hyaluronate receptor and actin filaments.

The association between actin and the receptor (whether direct or indirect) suggests that there is a transmembrane interaction between the hyaluronate receptor and actin filaments. This interaction may have important implications with respect to cell behavior. For example, the binding of hyaluronate to its receptor could serve as a signal causing actin filaments to organize or align in a particular pattern or conformation. This could explain the stimulatory effects of hyaluronate on neutrophil migration and phagocytosis (14, 15), and the role of hyaluronate in maintaining the proper morphology of developing salivary glands (2). Conversely, actin filaments could control the distribution of hyaluronate receptors on the cell surface and thereby regulate the adhesiveness of cells in different regions of the developing embryo. Due to its large and repetitive structure, a single molecule of hyaluronate can bind to a number of receptors clustered in one region of the cell surface. Indeed, a number of studies have shown that hyaluronate can interact with more than one receptor at a time resulting in increased affinity (37, 38). Such strong adhesion could then be weakened if the receptors were redistributed on the cell surface through cytoskeletal reorganization.

We are currently investigating whether the hyaluronate receptor is associated with actin filaments directly, or indirectly via interactions with other proteins such as talin, alpha-actinin, or vinculin. Preliminary data from our laboratory suggest that it is possible to isolate the receptor as part of a large complex from the membranes of both SV-3T3 and BHK cells. Biochemical dissection of this macromolecular complex may result in a better understanding of the exact transmembrane mechanism by which extracellular hyaluronate mediates intracellular processes.

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