Volume 336, number 3, 511–515 FEBS 13470 December 1993

Evidence for the existence of hyaluronectin-binding proteins in the plasma membranes

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Received 28 October 1993

This report documents for the first time the existence of specific binding proteins for hyaluronic acid binding protein (hyaluronectin) in the plasma membranes of normal and transformed cells. Firstly, we showed the specific binding of hyaluronic acid binding protein to the cell surface of normal rat heart fibroblasts (NRHF) by saturation and competition methods using ^{125}I-labeled hyaluronic acid binding protein and calculated the binding dissociation constant (0.43×10^{-13} M). In order to identify hyaluronectin-binding protein on the cell surface, plasma membranes isolated from rat brain, liver and fibrosarcoma were separated by SDS-PAGE and transferred to nitrocellulose paper by electroblotting. Incubation of the transferred membrane proteins with ^{125}I-labeled hyaluronectin in the presence of non-ionic as well as ionic detergents revealed two prominent bands of approximate molecular mass of 37 kDa and 40 kDa in brain, liver and fibrosarcoma. The specificity of the binding [^{125}I]hyaluronectin to 37-kDa and 40-kDa membrane proteins was further confirmed, as the intensity of the bands was reduced in the presence of a 20-fold excess of unlabeled hyaluronectin. We discuss our observations on hyaluronectin-binding membrane proteins in the context of hyaluronectin-mediated cellular functions.

Hyaluronic acid; Hyaluronectin; Plasma membrane; Hyaluronectin binding protein

1. INTRODUCTION

Cell–matrix and cell–cell interactions are fundamental requirements in a number of cellular functions such as proliferation, differentiation and transformation. Such interactions occur through specific cell surface receptor systems that bind to extracellular adhesive proteins, and provide these a putative transmembrane link to intracellular cytoskeletal components. Recently, a family of cell adhesion receptors, termed 'integrins', has been described that is functionally implicated in each of these biological events [1–3]. The integrins include the receptor of ECM proteins [4–9] and promote cell attachment to their extracellular ligands. Besides integrins, several other cell surface binding proteins or cell surface receptors for laminin and collagens have also been characterized from various cell types [2,10] and are implicated in cell–matrix or cell–cell interactions. Laminin binding proteins with higher affinity apparently consist of a single 67-kDa to 180-kDa glycoprotein, and are functionally as well as structurally distinct from integrin receptors [10].

Recently, we have reported the isolation and characterization of hyaluronectin, a homodimeric glycoprotein of 34-kDa subunits, as a principal binding site for hyaluronan in the extracellular matrices [11]. Its localization on the cell surface and involvement in the cell–substratum adhesion, as well as, its participation in the cell–cell interaction during solid tumor formation by transformed histiocytic macrophages has also been reported [12]. As with other ECM adhesive proteins discussed above, these biological effects of hyaluronectin on cells are thought to be mediated through specific cell-surface receptors or binding proteins in the plasma membranes.

In order to understand the mechanism of regulation of the cell functions mediated by hyaluronectin–cell interaction, we attempted to identify hyaluronectin-binding site on the cell surface.

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Abbreviations: HA, Hyaluronic acid; HABP, Hyaluronic acid binding protein; NRHF, Normal rat heart fibroblasts; ECM, Extracellular Matrix.

2. MATERIALS AND METHODS

2.1. *Materials*

Unless and otherwise mentioned, chemicals were purchased from Sigma Chemicals Co., St. Louis, USA. Molecular weight markers for SDS-PAGE and AH-Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. [^{125}I]Iodine was purchased from BARC, India. X-omat AR X-ray films were purchased from the Eastman Kodak Company, Rochester, NY 12650, USA.

2.2. *Preparation of plasma membranes*

Plasma membranes were isolated from rat liver essentially following the method of Ray [13], using discontinuous sucrose density gradient composed of 45%, 41% and 37% sucrose (w/w). Rat brain plasma membranes were prepared according to Havrankova and Roth [14] and Hajos [15] using discontinuous sucrose density gradient made up

of 1.2 M and 0.8 M sucrose (w/w). Plasma membrane of ascitic fibrosarcoma cells were isolated over a 41% sucrose solution according to Maeda et al. [16]. Liver plasma membranes from the 37% to 41% sucrose interface and brain plasma membranes from the 0.8 to 1.2 M sucrose interface and fibrosarcoma plasma membranes as white interfacial band were collected and washed with 0.01 M phosphate-buffered saline (PBS), pH 7.2 and stored at −70°C.

2.3. *Purification of hyaluronectin and protein labeling*

68-kDa hyaluronectin was purified from adult rat kidney according to our previous method using ammonium sulphate fractionation and hyaluronate-sepharose chromatography [11]. 68-kDa hyaluronectin was radiolabeled with [^{125}I]iodine to a specific activity (1.4×10^5 cpm/μg of protein for blotting experiments and 1.09×10^3 cpm/μg of protein for binding assay), using the Iodogen method described by Fraker and Speck [17].

2.4. *Binding of [^{125}I]hyaluronectin to cells (hyaluronectin–cell binding assay)*

Hyaluronectin–cell binding assays were performed with NRHF cells grown in monolayers using radiolabeled ligand, i.e. ^{125}I-labeled hyaluronectin following the method of Turley et al. [18] with some modifications.

The NRHF were plated on to 24-well tissue culture plastic plates (Coster) at a density of 1×10^6 cells per well in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The fibroblasts were incubated in this medium for 24 h at 37°C and 5% CO_2 until monolayers formed in the wells. The monolayers were incubated with 0.2 M urea in the same medium containing 1% FCS for 24 h at 37°C to expose the binding sites of hyaluronectin. The cells were then washed several times in DMEM containing cycloheximide (0.2 μg/ml) to prevent resynthesis of surface materials removed by urea treatment [19]. Before the binding assay, the fibroblast monolayers pretreated with urea were blocked by incubation with 0.1% BSA in serum-free DMEM for 2 h at 37°C. This was followed by incubation with increasing amounts (2 to 30 ng/ml per well) of [^{125}I]hyaluronectin (specific activity 1.09×10^3 cpm/ng of protein) in serum-free DMEM containing 0.2 μg cycloheximide/ml for 20 min at 37°C. In all cases the final volume of incubation mixture was kept 1.0 ml/well. Identical binding studies were also carried out using NRHF monolayers that were not treated with urea. After binding incubation, unbound [^{125}I]hyaluronectin was removed by washing the cells four times with serum-free DMEM. The cells were then solubilized in 0.1 M NaOH. Both cell-bound and unbound radioactivity were counted in a gamma-counter. The cell protein was measured by Lowry et al. [20].

Specificity of the binding of [^{125}I]hyaluronectin was tested by the competition of radiolabeled hyaluronectin with excess unlabeled hyaluronectin for binding. For the competition assay, a fixed amount of [^{125}I]hyaluronectin (i.e. the concentration at saturation level, 28 ng/ml/well) and increasing amounts (5- to 50-fold excess) of unlabeled hyaluronectin were added to the cells, and the decrease in binding of [^{125}I]hyaluronectin with increasing competition by unlabeled hyaluronectin was measured with the above protocol.

2.5. *Western blotting of plasma membranes with ^{125}I-labeled hyaluronectin*

To identify hyaluronectin-binding proteins, Western blotting of plasma membranes of brain, liver and fibrosarcoma with [^{125}I]hyaluronectin was performed according to Lesot et al. [21]. Purified liver, brain, and fibrosarcoma plasma membrane proteins were separated in 0.1% SDS on 12.5% polyacrylamide slab gel using discontinuous buffer system described by Laemmli [22] and then electroblotted on nitrocellulose sheets [23]. After washing briefly in 0.01 M PBS, pH 7.2 containing 0.05% Tween-20 (PBST), the sheets supporting transferred materials were saturated with 3% BSA in PBST at 37°C for 2 h. The sheets were washed with PBST and incubated with [^{125}I]hyaluronectin (sp. activity 1.4×10^5 cpm/μg of hyaluronectin) in PBS containing 1% BSA and 0.05% Tween-20 overnight at room temperature. After washing all the NC sheets exhaustively with PBST, the NC sheets were dried and exposed to Kodak X-omat AR films for autoradiography. Specific binding of [^{125}I]hyaluronectin to membrane proteins was tested by adding a 20-fold excess of unlabeled hyaluronectin to the incubation solution.

In order to eliminate non-specific or low-specificity binding, [^{125}I]hyaluronectin incubations and washings were performed under conditions of elevated stringency, i.e. using PBS containing Na-deoxycholate (ionic detergent).

3. RESULTS

To characterize the mode of association of extracellular 68-kDa hyaluronectin with the cell surface, we studied the binding of hyaluronectin to the cell surface of intact normal rat heart fibroblasts (NRHF) by the saturation and competition methods using ^{125}I-labeled hyaluronectin. As shown in Fig. 1a [^{125}I]hyaluronectin, added in increasing amounts (i.e. 2 to 30 ng per well in serum-free DMEM) to intact urea-pretreated or untreated monolayers of NRHF, bound in a linear and

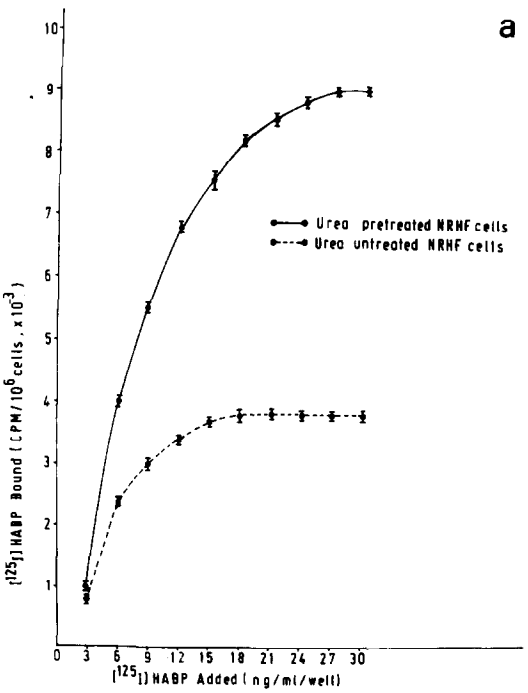

Fig. 1(a). Saturation of binding of [^{125}I]hyaluronectin to intact urea-pretreated or untreated normal rat heart fibroblasts (NRHF) monolayers. The assay of [^{125}I]hyaluronectin binding to NRHF was carried out as described in section 2. The monolayer cultures (approximately 10^6 cells/well) were pretreated with 0.2 M urea to expose cell-surface binding sites for hyaluronectin (●—●), or with culture medium only (●---●). Monolayers were washed in DMEM containing 0.2 μg/ml of cycloheximide, and then incubated with increasing amounts of [^{125}I]hyaluronectin (specific activity 1.09×10^3 cpm/ng of protein) in serum-free DMEM for 90 min at 37°C. After binding incubation, the monolayers were washed, solubilized in 0.1 M NaOH, and then counted for the cell-bound radioactivity in a gamma-counter. The cell-bound radioactivity (i.e. cell-bound [^{125}I]hyaluronectin in cpm/10^6 cells) is plotted as a function of the amount of the added radiolabeled hyaluronectin. Values represent the mean ± S.D. of six replicates from one assay. The entire assay was repeated at least twice with similar results. Hyaluronectin has been termed as HABP in photograph.

saturable manner. However, [^{125}I]hyaluronectin bound in greater amounts to urea pretreated than to untreated fibroblasts (Fig. 1a), implying that the urea treatment exposed binding sites for hyaluronectin on the cell surface.

Scatchard plot [24] analysis of the binding of [^{125}I]hyaluronectin to intact urea pretreated and untreated NRHF demonstrated only a single class of binding sites for hyaluronectin on the surface of the cells with apparent dissociation constants (K_d) of 0.43×10^{-13} M and 2×10^{-13} M respectively (Fig. 1b).

The specificity of [^{125}I]hyaluronectin binding to these cells was confirmed by the observation that the addition of increasing amounts of unlabeled hyaluronectin along with a constant amount of [^{125}I]hyaluronectin progressively reduced the binding of [^{125}I]hyaluronectin to the cells, which further confirms the specificity of the binding (Fig. 2). In order to identify the specific hyaluronectin binding protein, the plasma membrane proteins of brain, liver and fibrosarcoma were prepared by sucrose density gradient. The proteins were separated by SDS-PAGE and transferred to nitrocellulose paper by electroblotting. The plasma membrane hyaluronectin acid binding protein has been identified by overnight incubation of this nitrocellulose paper with [^{125}I]hyaluronectin in the presence of non-ionic detergent with or without excess of unlabeled hyaluronectin.

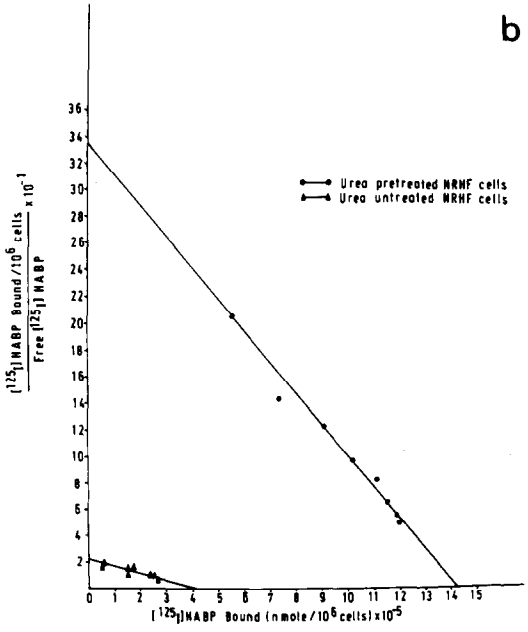

Fig. 1(b). Scatchard analysis of the binding of [^{125}I]hyaluronectin to urea-pretreated and untreated NRHF cells. The binding data for urea-pretreated (●—●) and untreated (▲—▲) cells (obtained in the absence of unlabeled hyaluronectin) were analyzed by plotting the ratio of the bound concentration of radiolabeled hyaluronectin to the free concentration as a function of the bound concentration of radiolabeled hyaluronectin. The scatchard plot analysis was used to determine K_d (slope) for the hyaluronectin binding to urea pretreated and untreated fibroblasts. Hyaluronectin has been termed as HABP in photograph.

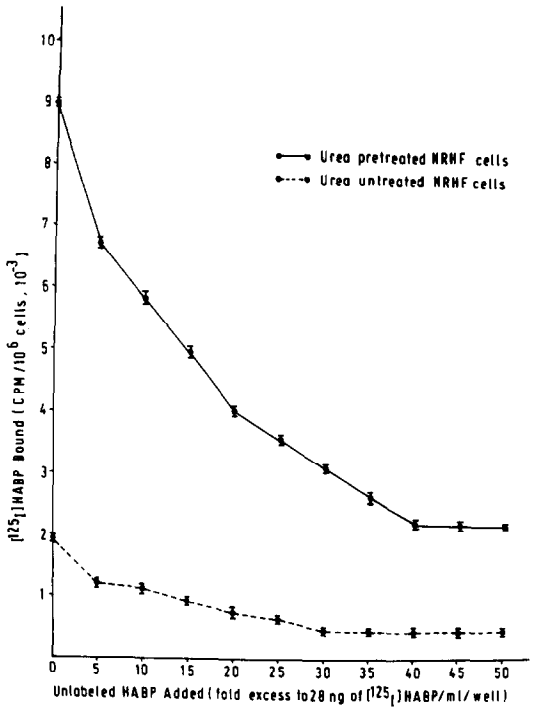

Fig. 2. Inhibition of binding of [^{125}I]hyaluronectin to intact urea-pretreated or untreated NRHF monolayers by increasing amounts of unlabeled hyaluronectin. The competition binding assays were performed as described in section 2. The intact urea-pretreated or untreated monolayer cultures of NRHF (approximately 10^6 cells/well) were incubated with a constant amount (28 ng/ml/well) of [^{125}I]hyaluronectin plus increasing amounts (5- to 50-fold excess) of unlabeled hyaluronectin in serum-free DMEM for 90 min at 37°C. The monolayers were then washed, solubilized in 0.1 M NaOH, and subsequently counted for cell-bound radioactivity in a gamma-counter. The cell-bound radioactivity (i.e. cell-bound [^{125}I]hyaluronectin in cpm/10^6 cells is plotted as a function of amount of the added unlabeled hyaluronectin. Values represent the mean ± S.D. of six replicates from one assay. The entire assay was repeated at least twice with similar results. (●—●), (●---●) Urea-pretreated and untreated NRHF cells, respectively. Hyaluronectin has been termed as HABP in the photograph.

After extensive washing with non-ionic detergent, two prominent bands of 37 kDa and 40 kDa were detected after autoradiography (Fig. 3A and B). Binding of [^{125}I]hyaluronectin to these protein bands was suppressed in the presence of a 20-fold excess of the unlabeled hyaluronectin, which suggested that the binding was specific (Fig. 3A, lanes 3 and 5, Fig. 3B, lane 3). Further, when hyaluronectin incubation and washings were performed under elevated ionic strength (i.e. in the presence of ionic detergent), the [^{125}I]hyaluronectin still bound specifically to these membrane protein bands at 37 kDa and 40 kDa (Fig. 3A, lane 4, Fig. 3B, lane 2). Preferential binding of [^{125}I]hyaluronectin to 37-kDa and 40-kDa membrane proteins even in the presence of ionic detergent and its reduced affinity in the presence of the excess unlabeled hyaluronectin confirm that 37-kDa and 40-kDa membrane proteins are specific hyaluronectin binding proteins in the plasma membrane of rat brain, liver cell and fibrosarcoma. As results are

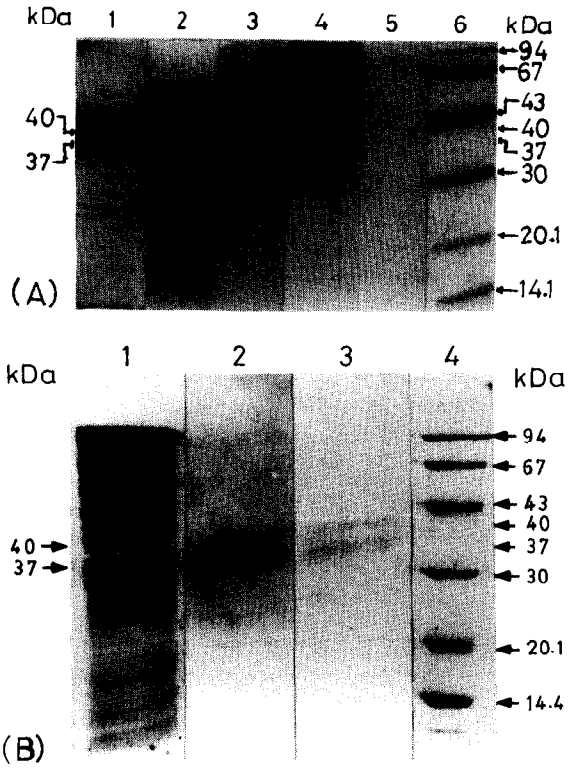

Fig. 3A and B. Binding of ^{125}I-labeled 68-kDa hyaluronectin to plasma membrane proteins of rat brain (A) and fibrosarcoma (B), plasma membranes were collected on a sucrose gradient and separated by 12.5% SDS-PAGE. After transfer to NC paper by electroblotting, sheets were incubated with [^{125}I]hyaluronectin, washed and autoradiographed as mentioned in section 2. Lanes A1 and B1, total plasma membrane proteins of rat brain (A1) and fibrosarcoma (B1) after electrophoresis and staining with Coomassie blue. Lanes 2–5 (A), autoradiograph after staining and incubation with [^{125}I]hyaluronectin shows preferential binding of [^{125}I]hyaluronectin to two membrane proteins of 37 kDa and 40 kDa in the presence of non-ionic detergent without excess of unlabeled hyaluronectin (A2); non-ionic detergent with a 20-fold excess of unlabeled hyaluronectin (A3); ionic detergent without excess of unlabeled hyaluronectin (A4); ionic detergent with a 20-fold excess of unlabeled hyaluronectin (A5). Lane B2, non-ionic and ionic detergent without excess of unlabeled hyaluronectin. Non-ionic and ionic detergent with a 20-fold excess of unlabeled hyaluronectin (B3). Low molecular weight markers (lanes A6 and B4) were stained with Amido black staining.

the same for liver and brain, no separate photograph is given for the liver experiment.

4. DISCUSSION

In recent years, rapid progress has been made in understanding the molecular interactions that result in cell adhesion. It is becoming clearer only now that extracellular adhesive proteins and their specific cell surface receptors play an important role in cell–matrix and cell–cell adhesion in many physiologically important processes including oncogenic transformation, and are key components of the cellular communication network. Thus, in this context the present study documents the following important observations: (a) linear and saturable binding of exogenous [^{125}I]hyaluronectin to normal rat heart fibroblasts demonstrating the existence of specific binding sites for this protein on cell membrane; and finally (b) the identification of hyaluronectin binding proteins of molecular mass of 37 kDa and 40 kDa in the plasma membrane of brain, liver and fibrosarcoma.

The 37-kDa and 40-kDa membrane components are shown to be specific hyaluronectin-binding proteins, as [^{125}I]hyaluronectin binds to these membrane proteins even in the presence of ionic detergents. Moreover, the reduced binding of [^{125}I]hyaluronectin to 37-kDa and 40-kDa membrane proteins in the presence of a 20-fold excess of unlabeled hyaluronectin confirms the specificity of the binding of hyaluronectin to these proteins. This observation is supported by our early report [12] that hyaluronectin stimulates cell–substratum attachment which can be specifically blocked by its antibody. It is important to mention that hyaluronectin-mediated cell–substratum attachment is independent of its ligand, i.e. HA. The crucial role of hyaluronectin–cell interaction in cell–cell adhesion was further evident from our previous finding [12] that pretreatment of virally transformed histiocytic macrophages with anti-hyaluronectin antibodies abolished their capacity to grow as solid tumors. In the light of the cell adhesive property [12] of hyaluronectin, our present results on the existence of hyaluronectin-binding proteins in the plasma membranes perhaps may shed light on the mechanisms by which hyaluronectin interacts with cells and then mediates physiologically important processes especially those which involve transmembrane signal transmission.

The presence of 37-kDa and 40-kDa binding proteins for hyaluronectin in plasma membrane is further justified as there are increasing evidences for the existence of integral membrane proteins in small molecular weight range in various cell types and tissues, which are involved in cell–matrix or cell–cell interaction and are distinct from integrins [2]. The smaller sized and monomeric collagen-binding membrane proteins ranging in size from 31-kDa to 37-kDa have been identified more recently [2]. It is interesting that the affinity of hyaluronectin binding protein for hyaluronectin is similar to the 67-kDa to 70-kDa monomeric laminin-binding protein, which also have high affinity for laminin, binding well even after solubilization in SDS and blotting onto nitrocellulose [10,21].

Although our data clearly show the existence of specific hyaluronectin binding proteins in the plasma membranes of rat brain, liver and fibrosarcoma, they are fragmentary, and do not yet indicate the homology of these hyaluronectin-binding membrane proteins with the known cell surface receptors for binding proteins for extracellular adhesive proteins. A precise structural comparison can be made in future only after the isolation and characterization of hyaluronectin binding protein.

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