Detergent Solubilization of the Endocytic Ca^{2+}-Independent Hyaluronan Receptor from Rat Liver Endothelial Cells and Separation from a Ca^{2+}-Dependent Hyaluronan-Binding Activity†

Judith Yannariello-Brown and Paul H. Weigel*

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

Received July 29, 1991; Revised Manuscript Received October 4, 1991

ABSTRACT: Rat liver sinusoidal endothelial cells (LECs) mediate the removal of hyaluronan (HA) from the circulation via a specific Ca^{2+}-independent endocytic receptor. To characterize the receptor biochemically, detergent-soluble extracts were prepared from crude LEC membranes. Using a dot blot assay to quantitate ^{125}I-HA binding activity in CHAPS-solubilized membranes, we detected not only specific Ca^{2+}-independent but also specific Ca^{2+}-dependent HA-binding activity. Both HA-binding activities behave as integral membrane-associated proteins; they are not released from LEC membranes by treatment at pH 11, and they require detergent for extraction. The Ca^{2+}-independent HA receptor was inactivated by treatment at 56 °C for 30 min or with 200 mM DTT at 4 °C for 30 min, whereas the Ca^{2+}-dependent activity actually increased by 75% after treatment at 56 °C and only 20% of the Ca^{2+}-dependent activity was lost after DTT treatment. A two-cycle membrane extraction protocol using CHAPS partially separated the two HA-binding activities. Eight millimolar KCl and 0.5% CHAPS extracted ~50% of the Ca^{2+}-independent HA receptor, but only 4–11% of the Ca^{2+}-dependent activity. When the KCl and CHAPS concentrations were increased to 2.0 M and 1.5%, respectively, the remaining HA receptor, as well as ~89–96% of the Ca^{2+}-dependent activity, was then extracted. The Ca^{2+}-independent and Ca^{2+}-dependent activities could also be further separated using Sephacryl S-400 gel filtration chromatography. The HA receptor activity eluted with a $M_r \sim 1 \times 10^6$ in buffer with 8 mM KCl and 0.5% CHAPS, but was resolved into additional smaller peaks when the CHAPS and KCl concentrations were increased to 1.5% and 2.0 M, respectively. In the same extract the elution pattern of the Ca^{2+}-dependent activity was only slightly affected (M_r shifted from 66×10^3 to 90×10^3) by the presence of 2.0 M KCl and 1.5% CHAPS. The Ca^{2+}-independent and Ca^{2+}-dependent activities did not coelute under any condition tested. These results show that the Ca^{2+}-independent HA receptor can be solubilized with retention of activity and that it is not only functionally distinct, but also biochemically and physically distinct from a Ca^{2+}-dependent HA-binding activity.

The nonsulfated glycosaminoglycan, hyaluronan (HA),[1] is composed of repeats of the disaccharide unit β-(1,4)-D-glucuronic acid-(β1,3)-*N*-acetyl-D-glucosamine (Hascall & Hascall, 1981). HA is important in the regulation of many biological processes such as cell migration and locomotion (Turley et al., 1990; Underhill & Dorfman, 1978), the regulation of immune responses (Forrester & Wilkinson, 1981), development (Toole, 1981), cell division (Brecht et al., 1986), wound healing (Weigel et al., 1986; Bertolami, 1984), and tumorigenesis and metastasis (Knudson et al., 1989; Turley, 1984). HA is found ubiquitously distributed throughout all vertebrate tissues and is particularly abundant in soft connective tissues, vitreous humor, and synovial fluid (Balazs et al., 1967). The normal turnover rate of HA in vivo is remarkably high, even for such an abundant molecule. The average, healthy adult human will turnover about 4 g of HA/day (Fraser & Laurent, 1989). The majority of the extracellular HA fragments are efficiently removed from the tissues by the lymphatic system (Fraser et al., 1988), and the remainder enters the blood and is removed by the sinusoidal endothelial cells of the liver via a class II endocytic recycling receptor (Eriksson et al., 1983; Raja et al., 1988; McGary et al., 1989). This receptor has a relatively high affinity for HA ($K_d = 10^{-11}$–10^{-8} M, depending on the HA size) and mediates the efficient clearance of HA from the bloodstream. The receptor is also specific for other GAGs such as chondroitin sulfate (Raja et al., 1988; McGary et al., 1989; Smedsrod et al., 1984) and heparin (Raja et al., 1988; McGary et al., 1989). The function of the endocytic HA receptor in LECs is to remove HA and other GAGs from the circulation. The receptor mediates the endocytosis of, and allows the LECs to subsequently degrade, these polysaccharides. Failure to remove these molecules from the blood could lead to numerous physiological problems, including high blood pressure due to increased blood viscosity, suppression of the immune response, and increased chance of thromboembolic diseases (Evered & Whelan, 1989).

Because of its wide tissue distribution, HA interacts with many extracellular molecules. For example, HA binds to plasma fibrinogen (LeBoeuf et al., 1986; Frost et al., 1988) and other acute-phase proteins (Hutadilok et al., 1988) and a serum-derived hydrophobic 85-kDa HA-binding protein (Yoneda et al., 1990). HA interacts with other extracellular

† This work was supported by NIH Grant GM 35978 (P.H.W.), a James W. McLaughlin Foundation Post-Doctoral Fellowship (J.Y.-B.), and a National Research Service Award (AG 05492 J.Y.-B.).

* To whom correspondence should be addressed.

[1] Abbreviations: HA, hyaluronan (hyaluronate, hyaluronic acid); LEC, liver endothelial cell; BSA, bovine serum albumin; GAG; glycosaminoglycan; PBS, phosphate-buffered saline; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DOC, deoxycholate; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; cc, correlation coefficient.

0006-2960/92/0431-576$03.00/0

matrix proteins such as cartilage link protein and proteoglycans (Hascall, 1977). HA-binding proteins or receptors are also found on the surface of many different cell types. For example, macrophages have HA receptors (Gustafson & Forsberg, 1991; Green et al., 1988) and oligodendrial cells bind HA via the cell surface molecule hyaluronectin (Delpech et al., 1976). Subpopulations of B and T lymphocytes express the hematopoietic form of CD44, a surface HA-binding glycoprotein (also called Pgp-1 or Hermes) which is thought to be a lymphocyte homing receptor (Stamenkovic et al., 1991). Other cell surface HA receptors include a glial cell HA-binding protein (Perides et al., 1989), an 85-kDa protein in Swiss mouse embryo 3T3 and SV3T3 transformed fibroblasts (Underhill & Toole, 1980) that is also a member of the CD44 family of molecules (Aruffo et al., 1990), a 50-kDa protein on mobile chicken embryo heart fibroblasts (Turley et al., 1990), a Ca^{2+}-dependent extracellular activity in LECs (Yannariello-Brown et al., 1992), and the class II endocytic LEC HA receptor (Smedsrod et al., 1984; Eriksson et al., 1983; Raja et al., 1988; McGary et al., 1989). Liver, in addition, has a 60-kDa HA-binding protein (D'Souza & Datta, 1985), whose localization is unknown, and hepatocytes have a predominantly intracellular HA/GAG-binding protein (Frost et al., 1990).

We have previously characterized a Ca^{2+}-dependent HA-binding activity in cultured LECs that is functionally distinct from the Ca^{2+}-independent endocytic receptor (Yannariello-Brown et al., 1992). This conclusion was based on two observations. First, the endocytic HA receptor in intact LECs does not require Ca^{2+} for binding or subsequent endocytosis of HA. Second, the endocytic receptor is localized both on the cell surface and internally, consistent with the fact that it recycles (McGary et al., 1989), whereas the Ca^{2+}-dependent HA-binding activity localized to the cell surface only. The biological function(s) of the Ca^{2+}-dependent HA-binding activity is (are) presently unknown. Since the Ca^{2+}-dependent activity is localized predominately, if not exclusively, extracellularly, it may play a role in cell adhesion or migration in response to local changes in Ca^{2+} concentration. Another function might be the organization of a HA-containing matrix, a role similar to that of the cartilage link protein (Hascall, 1977). Little biochemical information is available regarding the endocytic LEC HA receptor or the Ca^{2+}-dependent HA-binding activity. To determine if there is a relationship between the Ca^{2+}-independent endocytic HA receptor and the Ca^{2+}-dependent HA-binding activity, and to characterize further the Ca^{2+}-independent endocytic receptor, both activities were solubilized from LEC membranes under conditions that retain their ability to bind HA. Comparison of the biochemical and physical properties of these two activities shows that they differ biochemically and physically.

Experimental Procedures

Materials. $Na^{125}I$ was purchased from Amersham Corp. (10–20 Ci/μg of iodine). 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (Iodogen) was purchased from Pierce. Nitrocellulose paper (0.45 μm) was from Schleicher and Schuell. DTT was from Research Organics. BSA (fraction V) was from Armour Biochemicals. Collagenase was from Sigma (type I) and from Boehringer Mannheim Biochemicals (type D). Sephacryl S-400 was from Pharmacia. Nonidet P-40 was from United States Biochemical Corp. Econo-columns were from Bio-Rad. Teflon pestles were from Kontes. Tissue culture reagents were from Gibco. HA and all other chemicals and reagents were from Sigma.

General Methods. ^{125}I radioactivity was determined using a Packard Multiprias 2 γ spectrometer. Protein was determined by the methods of Bradford (1976) or Lowry et al. (1951), depending upon the salt and/or detergent present in the sample, using BSA as a standard. The experimental points represent the average of duplicates. SEM bars are included for all data points unless the symbols were larger than the SEM, in which case they are not shown.

Purification and Iodination of HA. Human umbilical cord HA was further purified by complexation with cetylpyridinium chloride, fractionation on Celite, and ethanol precipitation (Scott, 1960). The purified HA contains <0.2% protein and <0.1% sulfate by weight. HA oligosaccharides averaging 80000 Da (~400-mer) were obtained from a specific lot supplied by Sigma and were used without further fragmentation. As previously described (Raja et al., 1984) with minor modifications (Frost et al., 1988), HA oligosaccharides, which normally do not have free amino groups, were uniquely modified at the reducing end of the molecule to produce an HA–hexylamine derivative. This unique modification allows a subsequent reaction with the Bolton–Hunter reagent to produce a hydroxyphenyl derivative that was iodinated with $Na^{125}I$ using Iodogen as described by Raja et al. (1984). We routinely obtain a specific activity of 150 cpm/fmol of HA.

Isolation of LEC. Male Sprague–Dawley rats were purchased from Harlan Breeding Laboratories, Houston, TX, and used between 6 and 10 weeks of age. The method of Eriksson et al., (1983) was utilized for LEC isolation with minor modifications (McGary et al., 1989). Rat livers were perfused with collagenase as previously described (Oka & Weigel, 1987), and the nonparenchymal cell fraction was collected from the first three differential centrifugations. These cells were pooled, centrifuged, and resuspended in medium 1/BSA, which contains Eagle's modified minimum essential medium (Gibco) supplemented with 2.4 g/L HEPES, pH 7.4, 0.22 g/L $NaHCO_3$, and 0.15% BSA (w/v). The LECs were then further purified by centrifugation through a discontinuous Percoll gradient prepared in PBS. Cells banding at the 25/50% interface were removed and washed 3 times in PBS at 4 °C. For the last centrifugation, the cells were placed in a 15-mL conical tube in order to measure the pellet volume.

Preparation of LEC Membranes. Membranes were prepared from freshly isolated LECs according to a protocol previously described for large-vessel endothelial cells (Yannariello-Brown et al., 1988). Ten volumes of hypotonic buffer containing 10 mM Tris, pH 8.0, 5 mM EGTA, 2 mM $MgCl_2$, and a protease inhibitor cocktail (final concentrations of 2 mM DFP, 1.5 mM PMSF, and 1.5 mM NEM) were added to the cell pellet on ice and incubated for 15 min. The cell suspension was then disrupted with ~10 strokes of a Dounce homogenizer fitted with a size A pestle. Samples were taken every 5 strokes and viewed by phase contrast microscopy to monitor cell breakage and nuclear integrity. If at any time during the preparation >10% of the nuclei were disrupted, the preparation was discarded. When ~80% of the cells were disrupted, 3 volumes of hypotonic buffer containing 0.3 M sucrose was added and unbroken cells and nuclei were removed by centrifugation at 1000g for 5 min. The supernatant was saved and the pellet was rehomogenized with 10–20 strokes of a B pestle in the original volume of hypotonic buffer to break up the pellet and release any trapped membranes. The homogenate was centrifuged as before, and the resulting supernatants were pooled and centrifuged at 105000g for 60 min to pellet the crude membrane fraction. The resulting membranes were either used immediately or frozen at −80 °C in hypotonic buffer containing 10% glycerol and a protease inhibitor cocktail.

Detergent Solubilization. All membrane extractions were performed in buffer 1 (10 mM HEPES, pH 7.5, 8 mM KCl, and 140 mM NaCl) containing 5 mM EDTA, a detergent, and the above protease inhibitor cocktail. The type of detergent, the detergent to protein ratio, KCl concentration, and the number of extraction cycles were varied depending on each individual experiment. Two different protocols were used to extract ^{125}I-HA binding activity from LEC membranes.

(A) One-Cycle Extraction. LEC membranes were extracted in buffer 1 containing 5 mM EDTA, 1.0 M KCl, a detergent, and the above protease inhibitor cocktail by homogenizing with a Teflon pestle and then gently stirring for 2 h on ice. The suspensions were centrifuged at 105000*g* for 60 min at 4 °C, and the detergent-soluble phase was removed. The residual insoluble pellet was then solubilized in 0.3 N NaOH and assayed for protein content. The detergent-soluble phase was assayed for protein content and ^{125}I-HA binding activity using the dot blot assay.

(B) Two-Cycle Extraction. LEC membranes were extracted in buffer 1 containing 5 mM EDTA, 0.5% CHAPS, and the protease inhibitor cocktail by homogenizing with a Teflon pestle and then gently stirring for 2 h on ice. The detergent to protein ratio during this cycle was 1:1. After extraction, the suspensions were centrifuged at 105000*g* for 60 min at 4 °C and the detergent-soluble phase was removed. We designate this the "low-KC" extract. The residual insoluble material was then reextracted for 2 h at 4 °C in buffer 1 containing 5 mM EDTA, 1.5% CHAPS, 2.0 M KCl, and the protease inhibitor cocktail at a detergent to protein ratio of 10:1. After extraction, the suspensions were centrifuged at 105000*g* for 60 min at 4 °C and the detergent-soluble phase was removed. This is designated the "high-KC" extract. The residual insoluble material was extracted in 0.3 N NaOH and assayed for protein content. The detergent-soluble material was assayed for protein content and ^{125}I-HA-binding activity using the dot blot assay.

Dot Blot Assay To Measure ^{125}I-HA-Binding Activity. The dot blot assay is based on a previously described assay for measuring ^{125}I-HA-binding activity in DOC-solubilized hepatocyte membranes (Frost et al., 1988), with modifications. Increasing concentrations of solubilized membrane proteins were spotted in duplicate onto nitrocellulose sheets using a dot blot apparatus (Schleicher and Schuell). The nitrocellulose sheets were incubated in buffer 1 containing 5% BSA for 2 h at 23 °C and either used immediately or stored overnight in the same buffer at 4 °C. To assess specific ^{125}I-HA-binding activity, identical sheets were incubated with 4 μg of ^{125}I-HA/mL in buffer 1 plus or minus a 125-fold excess of unlabeled HA for 2 h at 23 °C. At the end of the incubation, the nitrocellulose sheets were washed quickly 3 times in cold buffer 1 and once in buffer 1 containing 0.01% Nonidet P40 and then rinsed again in buffer 1. Each individual spot was cut out with a cork borer, and the radioactivity was quantitated. Specific cpm bound were determined by subtracting the cpm bound in the presence of excess unlabeled HA (nonspecific counts bound) from the cpm bound in the absence of unlabeled competitor (total counts bound). The data are presented as femtomoles of ^{125}I-HA bound. Specific activities (fmol of HA bound/mg of protein) were calculated from slopes determined by least-squares linear regression analysis. The Ca^{2+}-dependent activity was calculated by subtracting the specific ^{125}I-HA binding in the presence of EDTA from the specific binding in the presence of 10 mM $CaCl_2$. This estimates the Ca^{2+}-dependent activity without the contribution of the Ca^{2+}-independent activity.

The presence of excess detergent and/or high ionic strength in the extracts can adversely affect the ability to measure ^{125}I-HA binding activity using the dot blot assay. This is presumably due to interference with protein adsorption onto the nitrocellulose (Frost & Weigel, 1990; Smith et al., 1989). Therefore, prior to spotting samples on the nitrocellulose sheets, the detergent concentration was always adjusted to below the critical micelle concentration for the detergent used. For CHAPS extracts, the final concentrations were adjusted to 0.03% CHAPS and 40 mM KCl. If necessary, the dilution buffer used contained CHAPS or KCl to give these final concentrations in the diluted extract.

Results

Extraction of both Ca^{2+}-Independent and Ca^{2+}-Dependent ^{125}I-HA-Binding Activities from LEC Membranes. Initially, three separate classes of detergents (ionic, nonionic, and zwitterionic) were tested for their ability to solubilize the HA-binding activities from the LEC membranes using the one-cycle extraction protocol. Membrane aliquots were extracted with either 1.0% CHAPS, 1.0% Nonidet P-40, or 0.1% DOC in 1.0 M KCl. Using the dot blot assay, we determined that the ionic detergent DOC extracted slightly more total HA-binding activity than the zwitterionic detergent CHAPS and both of these detergents were far superior to the nonionic detergent Nonidet P-40. DOC extracts contained 1.4-fold more total HA-binding activity than CHAPS extracts and 4.7-fold more activity than Nonidet P-40. We chose to use the zwitterionic detergent CHAPS, rather than DOC, in all subsequent experiments for the following reasons: (1) CHAPS does not have a net charge; (2) CHAPS is soluble over a wide range of pH and ionic strength, (3) DOC can interfere with many SDS–PAGE and isoelectric focusing gel systems; and (4) since DOC has a negative charge, like HA, it might interfere with any subsequent affinity chromatography.

We have previously reported (Yannariello-Brown et al., 1992) the existence of two functionally distinct ^{125}I-HA-binding activities in cultured LECs. One activity is Ca^{2+}-independent and represents the class II endocytic HA receptor, and the second activity is a Ca^{2+}-dependent HA-binding activity that is localized extracellularly and does not mediate the internalization of HA. To determine if we could measure both HA-binding activities in detergent-soluble extracts of LEC membranes using the dot blot assay, crude LEC membranes were extracted with CHAPS (1.0%) using a one-cycle extraction procedure and increasing amounts of the extract protein were spotted onto nitrocellulose. ^{125}I-HA-binding activity was measured in the presence of either the divalent cation chelator EDTA or $CaCl_2$ to determine if the dot blot assay would detect both activities (Figure 1). In this experiment, the ^{125}I-HA binding measured with EDTA was not subtracted from the total activity obtained in the presence of $CaCl_2$. A linear increase in the total ^{125}I-HA-binding activity was observed in the immobilized extracts in the presence of 5 mM EDTA (2.8 fmol of HA/μg of protein; cc = 0.998) or the presence of 10 mM Ca^{2+} (6.2 fmol of HA/μg of protein; cc = 0.998); a 2-fold stimulation of total ^{125}I-HA binding activity was seen in the presence of 10 mM Ca^{2+}. The percentage specific binding ranged from 71% to 93% in the presence of Ca^{2+} and from 63% to 82% in the absence of Ca^{2+}. This result demonstrates that the dot blot assay can quantitate both Ca^{2+}-independent and Ca^{2+}-dependent HA-binding activities and that both activities are stable to detergent solubilization from LEC membranes.

Differential Extraction of the Two HA-Binding Activities. Experiments were carried out with CHAPS to determine the

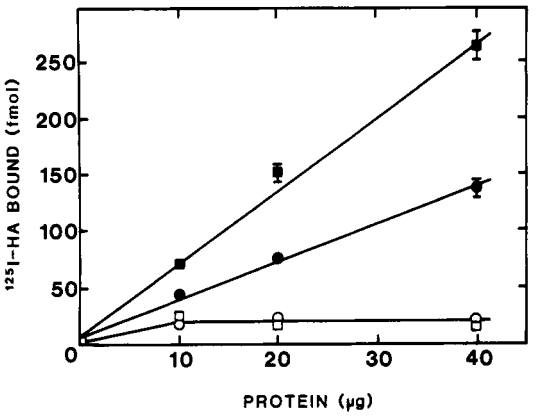

FIGURE 1: Quantitation of ^{125}I-HA-binding activity in detergent-soluble LEC membrane extracts using a dot blot assay. LEC membranes were extracted in buffer 1 containing 5 mM EDTA, 1.0% CHAPS, and 1.0 M KCl at a detergent to protein ratio of 2:1 using the one-cycle protocol described under Experimental Procedures. The detergent-soluble fraction was diluted 30-fold in buffer 1 to yield a CHAPS concentration of 0.03%, and the KCl concentration in the diluent was increased to 40 mM. Increasing concentrations of protein were then spotted onto four separate nitrocellulose sheets, and the sheets were incubated in the presence of 5 mM EDTA (●, ○) or 10 mM $CaCl_2$ (■, □) with 4 μg of ^{125}I-HA/mL in buffer 1 with (□, ○) or without (■, ●) a 125-fold excess of unlabeled HA to determine nonspecific and total ^{125}I-HA-binding activity, respectively. The lines, generated by least-squares linear regression analysis, had slopes of 6.2 fmol of HA/μg of protein in the presence of Ca^{2+} and 2.8 fmol of HA/μg of protein in the absence of Ca^{2+} (ccs = 0.998).

best extraction conditions for recovering the two HA-binding activities. A two-cycle extraction protocol was chosen that enriched for the Ca^{2+}-independent HA receptor in the first extraction. The first extraction was performed with buffer 1 containing 5 mM EDTA and a lower concentration of KCl and CHAPS, 8 mM and 0.5%, respectively, and is designated the "low-KC" extract. The second extraction was performed after increasing the KCl and CHAPS concentrations to 2.0 M and 1.5%, respectively, to give an extract designated as the "high-KC" extract. Using this protocol, ~64% of the total membrane protein was extracted. In 10 experiments, the low-KC extract contained ~70 ± 6% of the protein, and the high-KC extract contained ~30 ± 6% of the extractable protein. Samples of each extract were assayed for specific ^{125}I-HA binding in the presence of Ca^{2+} or the chelator EDTA; specific Ca^{2+}-dependent activity was calculated by subtracting the Ca^{2+}-independent activity from the activity with Ca^{2+} present as described under Experimental Procedures. The low-KC extract contained 30–65% of the total extractable Ca^{2+}-independent activity, but only 4–11% of the Ca^{2+}-dependent activity. The remaining extractable Ca^{2+}-independent activity, as well as >89% of the extractable Ca^{2+}-dependent activity, was recovered in the subsequent high-KC extract.

The results of two representative experiments are shown in Table I. The specific ^{125}I-HA-binding activity recovered in each extract was determined from the slope of activity versus protein concentration curves. The amount of specific HA-binding activity extracted per mg of membrane protein varied slightly between preparations. This was most likely due to variability of the starting LEC membranes and the length of time they were stored before extraction. The membranes will vary since they are isolated from primary LECs prepared by enzymatic digestion of intact liver. Figure 2 shows typical titration curves for the low-KC and high-KC extracts. The Ca^{2+}-independent HA receptor activity increased linearly with increasing protein and was 1.3 and 2.1 fmol of HA bound/μg of protein, respectively, in the low-KC and high-KC extracts

Table I: Enrichment of the Ca^{2+}-Independent HA Receptor Using a Two-Cycle Extraction Protocol[a]

	HA-binding activity					
	low-KC extract			high-KC extract		
expt no.	EDTA (fmol)	Ca^{2+} (fmol)	protein recovered (μg)	EDTA (fmol)	Ca^{2+} (fmol)	protein recovered (μg)
1	685	706	803	1191	5848	386
2	5741	7714	3588	3746	16056	1784

[a] LEC membranes were extracted at 4 °C for 2 h with buffer 1 containing 5 mM EDTA, 8 mM KCl, and 0.5% CHAPS (low-KC extract) followed by a second extraction in buffer 1 containing 5 mM EDTA, 2.0 M KCl, and 1.5% CHAPS (high-KC extract). The extracts were diluted with buffer 1 and 1 M KCl to yield final KCl and CHAPS concentrations of 40 mM and 0.03%, respectively. Increasing protein concentrations were spotted onto nitrocellulose, and specific Ca^{2+}-independent ^{125}I-HA binding was directly determined in the presence of 5 mM EDTA. Specific Ca^{2+}-dependent activity was calculated by subtracting the Ca^{2+}-independent activity from the specific ^{125}I-HA binding determined in the presence of 10 mM $CaCl_2$. The total sample HA-binding activity (fmol) was calculated from the specific binding activity of each sample (fmol of ^{125}I-HA bound/μg of protein), determined from the slope of the lines calculated by least-squares linear regression analysis. Forty-six milligrams and 220 mg of LEC membranes were extracted in experiments 1 and 2, respectively.

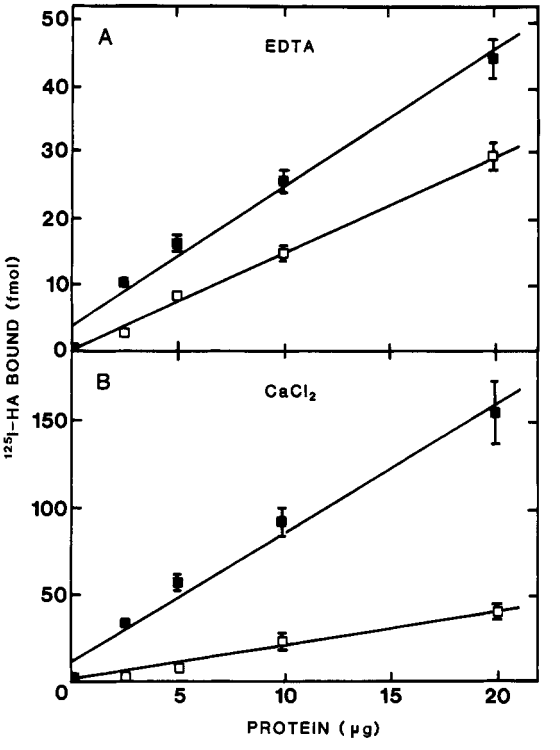

FIGURE 2: Partial separation of the Ca^{2+}-independent and Ca^{2+}-dependent HA-binding activities by sequential detergent extraction. LEC membranes were extracted using the two-cycle extraction protocol described under Experimental Procedures. The CHAPS and KCl concentrations were adjusted to 0.03% and 40 mM, respectively, in both the low-KC (□) and the high-KC (■) extracts before ^{125}I-HA-binding activity was determined by the dot blot assay. Specific Ca^{2+}-independent ^{125}I-HA-binding activity was determined in the presence of 5 mM EDTA (A). The specific Ca^{2+}-dependent ^{125}I-HA-binding activity was determined in the presence of 10 mM $CaCl_2$ (B) and was calculated by subtracting the Ca^{2+}-independent activity as described under Experimental Procedures.

(Figure 2A). The Ca^{2+}-dependent activity in the low-KC extract (1.9 fmol of HA bound/μg of protein) was much lower than the same activity in the high-KC extract (8.9 fmol of HA bound/μg of protein), reflecting the selective extraction of the Ca^{2+}-dependent activity into the high-KC extract. We have also performed two successive extractions with the low-KC

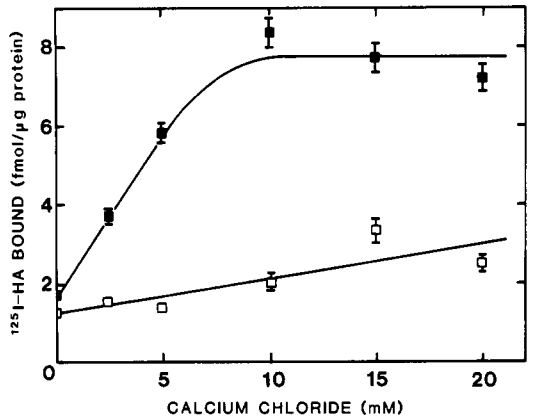

FIGURE 3: Effect of increasing $CaCl_2$ concentration on ^{125}I-HA-binding by the low-KC and high-KC extracts. After adjusting the CHAPS and KCl concentrations as described under Experimental Procedures, the final protein concentrations were 82 and 14 μg/mL, respectively in the low-KC and high-KC extracts. Ten micrograms of protein was immobilized onto each nitrocellulose spot, and specific ^{125}I-HA-binding activity was determined in the low-KC extract (□) and the high-KC extract (■) by the dot blot assay in the presence of 5 mM EDTA (0 mM $CaCl_2$) or increasing concentrations of $CaCl_2$. The Ca^{2+}-independent activity was not subtracted from the total value determined in the presence of $CaCl_2$. Lines were calculated by least-squares linear regression analysis (ccs > 0.98). The specific HA-binding activities, calculated from the initial slopes, were 6.6 fmol of HA/(μg·mM Ca^{2+}) for the high-KC extract and 0.86 fmol/(μg·mM Ca^{2+}) for the low-KC extract.

buffer, followed by one cycle in high-KC buffer in order to extract the majority of the Ca^{2+}-independent activity without any contaminating Ca^{2+}-dependent activity. Although ~80% of the Ca^{2+}-independent activity was recovered in the two low-KC extractions, this treatment also solubilized ~41% of the Ca^{2+}-dependent activity.

The Ca^{2+} dependence of the ^{125}I-HA-binding activity in the low-KC and high-KC extracts was measured in the presence of increasing concentrations of calcium chloride (Figure 3). The binding response to increasing concentrations of Ca^{2+} by the high-KC extract was linear up to 10 mM with a slope of 6.6 fmol of HA/(μg of protein·mM Ca^{2+}) (cc = 0.99), but above this concentration the activity plateaued. The extent of specific ^{125}I-HA binding was stimulated 4-fold at 10 mM Ca^{2+}, when compared to the 0 mM Ca^{2+} (5 mM EDTA) control. ^{125}I-HA binding to the low-KC extract, on the other hand, increased linearly up to 15 mM Ca^{2+} with a slope of only 0.86 fmol of HA/(μg of protein·mM Ca^{2+}) (cc = 0.83). A <2-fold increase in binding at 15 mM $CaCl_2$ was seen when compared to the 0 mM Ca^{2+} (5 mM EDTA) control. Thus, there is a >7.5-fold difference in the stimulation of specific HA-binding activity by Ca^{2+} between the low-KC and high-KC extracts. This result supports the conclusion that the Ca^{2+}-dependent activity preferentially partitions into the high-KC extract.

Alkaline Sodium Carbonate Treatment of LEC Membranes. Most, if not all, class II endocytic receptors examined to date are thought to be integral membrane proteins (Anderson & Kaplan, 1983). This conclusion has been made by either direct or deduced sequence analysis, membrane protein topology or detergent solubility studies, or the criterion of resistance to mild alkaline treatment (Fujiki et al., 1982). Integral membrane proteins anchored into the plasma membrane directly by transmembrane domains or indirectly by lipid anchors are resistant to carbonate treatment. If the endocytic HA receptor or the Ca^{2+}-dependent HA-binding activity are integral membrane proteins, they should remain with the membranes after treatment with 0.1 M sodium carbonate at pH 11. When LEC membranes were treated at pH 11 and then sequentially extracted in low-KC, followed by high-KC buffer, only 4% of the total Ca^{2+}-independent activity and no Ca^{2+}-dependent activity was detected in the carbonate wash (Table II). The Ca^{2+}-independent HA receptor partitioned between the low-KC (45%) and the high-KC (55%) extracts as seen before, and the Ca^{2+}-dependent activity was recovered predominately (99%) in the high-KC extract. The carbonate wash removed ~50% of the total protein, which resulted in a 2-fold increase in the specific HA-binding activity of the extracts prepared from these alkaline-treated membranes compared to control membranes treated in hypotonic buffer at pH 7.5.

Table II: Both the Ca^{2+}-Independent HA Receptor and the Ca^{2+}-Dependent ^{125}I-HA-Binding Activity Remain Membrane Associated after Mild Alkaline Treatment[a]

	HA-binding activity [fmol recovered (% of total)]		
type of activity	carbonate wash	low-KC extract	high-KC extract
Ca^{2+} independent	44 (4)	324 (30)	717 (66)
Ca^{2+} dependent	0 (0)	16 (1)	2236 (99)

[a] LEC membranes were treated with 0.1 M sodium carbonate, pH 11.0, for 30 min at 4 °C. The membranes were then centrifuged at 105000g for 60 min and extracted using the two-cycle extraction protocol. The CHAPS and the KCl concentrations in both extracts were then adjusted to 0.03% and 40 mM, respectively. The pH and KCl and CHAPS concentrations in the carbonate wash were also adjusted to match those in the adjusted extracts. Increasing amounts of protein were spotted onto nitrocellulose, and specific Ca^{2+}-independent ^{125}I-HA binding was directly determined in the presence of 5 mM EDTA. Specific Ca^{2+}-dependent activity was calculated by subtracting the Ca^{2+}-independent activity from the specific ^{125}I-HA binding determined in the presence of 10 mM $CaCl_2$. Total HA-binding activity (fmol) recovered from each sample was calculated from the specific HA-binding activities, determined from the slope of the lines calculated by least-squares linear regression analysis.

Differential Effect of Temperature on the Ca^{2+}-Independent and Ca^{2+}-Dependent HA-Binding Activities. Both the Ca^{2+}-independent and Ca^{2+}-dependent ^{125}I-HA-binding activities were measured in the low-KC and high-KC extracts after treatment at various temperatures (Figure 4). The Ca^{2+}-independent HA receptor present in the low-KC extract was very sensitive to temperature. A 50% loss of activity occurred after incubation for 30 min at 37 °C, and virtually all activity was lost after treating at ≥56 °C for 30 min. Temperature treatment had the identical effect on the Ca^{2+}-independent activity in the high-KC extract (data not shown). The Ca^{2+}-dependent activity in the high-KC extract, however, behaved very differently. This activity was stimulated at temperatures >23 °C, increasing the activity to 175% of control at 56 °C. Even after treating the high-KC extracts at 80 °C for 30 min, or 100 °C for 3 min, the Ca^{2+}-dependent activity only decreased to 50% of the control value. When the Ca^{2+}-dependent activity was assayed in temperature-treated low-KC extracts, a small but significant increase in ^{125}I-HA binding was seen up to 56 °C (not shown). The shape of the curve for this activity versus temperature was identical to that seen in the high-KC extract, but on a much reduced scale, reflecting the relatively small amount of Ca^{2+}-dependent activity present in the low-KC extract. Similar patterns of temperature sensitivity for both activities in the two extracts were observed if the proteins were first immobilized onto nitrocellulose before the temperature treatment (not shown).

Differential Effect of Reduction on the Two HA-Binding Activities. To assess whether disulfide bonds are important in maintaining HA-binding activity, both the low-KC and

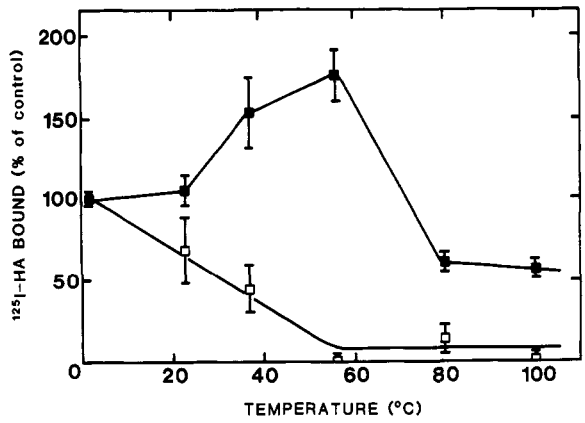

FIGURE 4: Temperature treatment differentially affects the Ca^{2+}-independent and Ca^{2+}-dependent HA-binding activities. After adjusting the CHAPS and KCl concentrations in the low-KC and high-KC extracts, the protein concentrations were 25 and 14 μg/mL, respectively. The diluted extracts were treated for 30 min at the indicated temperatures (3 min for the 100 °C point) and chilled to 4 °C, and aliquots containing 10 μg of protein were immobilized onto nitrocellulose spots to determine specific ^{125}I-HA-binding activity. Specific Ca^{2+}-independent activity (□) was determined in the presence of 5 mM EDTA in the low-KC extract. Specific Ca^{2+}-dependent activity (■) was calculated by subtracting the Ca^{2+}-independent activity (5 mM EDTA) from the specific ^{125}I-HA-binding activity determined in the presence of 10 mM $CaCl_2$ in the high-KC extract. ^{125}I-HA-binding activity in extracts incubated at 4 °C are designated as the controls, and all other values are expressed as the percent of the 4 °C control.

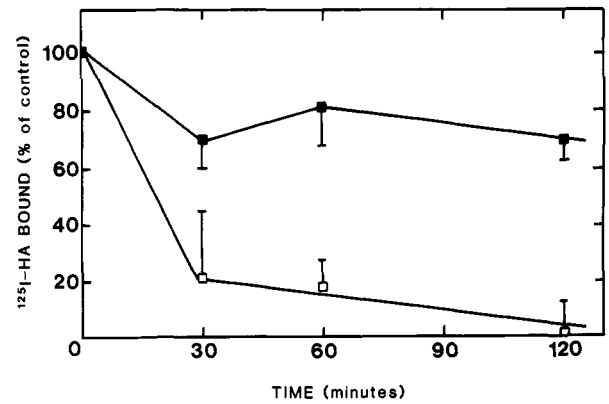

FIGURE 5: Reduction with DTT differentially affects the Ca^{2+}-independent and Ca^{2+}-dependent HA-binding activities. After the CHAPS and KCl concentrations were adjusted, the protein concentrations were 25 μg/mL in the low-KC extract and 14 μg/mL in the high-KC extract. Solid DTT was added to the extracts to give a final concentration of 200 mM. The extracts were incubated for the indicated time at 4 °C and then alkylated at 4 °C for 15 min using iodoacetamide at a final concentration of 10 mg/mL. After alkylation, 10 μg of protein was immobilized onto nitrocellulose spots, and specific ^{125}I-HA-binding activity was measured. The Ca^{2+}-dependent activity was measured in the high-KC extract (■), and the Ca^{2+}-independent activity was measured in the low-KC extract (□) as described for Figure 4. Control extracts were incubated for 120 min at 4 °C without DTT, but with alkylation. The data are expressed as the percent of the control value.

high-KC extracts were first diluted to give CHAPS and KCl concentrations of 0.03% and 40 mM, respectively. The adjusted extracts were then treated with 200 mM DTT at 4 °C for various times and alkylated, and then ^{125}I-HA-binding activity was measured by the dot blot assay (Figure 5). As controls, samples of both extracts were alkylated without reduction. Alkylation alone only resulted in a 5% decrease in the Ca^{2+}-independent activity and a 10% decrease in the Ca^{2+}-dependent activity, when compared to nonalkylated samples. Neither HA-binding activity, therefore, appears to require an essential free sulfhydryl group for activity. Preliminary experiments indicated that high DTT concentrations were required for reduction at 4 °C within 30 min. Since a concentration-dependent decrease in Ca^{2+}-independent HA-binding activity occurred in the range of 50–200 mM DTT, a concentration of 200 mM was chosen for the experiment in Figure 5.

Reduction and alkylation of the high-KC extract resulted in only a 20% decrease in specific Ca^{2+}-dependent ^{125}I-HA-binding activity even after treating for 120 min (Figure 5). However, reduction and alkylation of the low-KC extract resulted in an 80% loss of Ca^{2+}-independent activity by 30 min and a complete loss of activity by 120 min. The endocytic HA receptor is, therefore, inactivated by reduction, whereas the Ca^{2+}-dependent HA-binding activity is not.

Separation of the Ca^{2+}-Independent and Ca^{2+}-Dependent HA-Binding Activities by Gel Filtration Chromatography. The data thus far strongly suggest that the two ^{125}I-HA-binding activities are distinct molecules. To support this conclusion further, gel filtration chromatography on Sephacryl S-400 was used in an attempt to separate the two activities and partially purify the Ca^{2+}-independent HA receptor. The protein elution profiles of the low-KC and high-KC extracts were distinct (Figure 6A), indicating that a different size range of proteins had been extracted. The elution profiles for the HA-binding activities in the low-KC extract (Figure 6B) and high-KC extract (Figure 6C) were also different. The Ca^{2+}-independent HA receptor in the low-KC extract (Figure 6B) eluted with a K_{av} = 0.09 ($M_r > 1 \times 10^6$), while the Ca^{2+}-dependent HA-binding activity eluted with a K_{av} = 0.63 ($M_r \sim 66 \times 10^3$). The Ca^{2+}-dependent activity in the high-KC extract (Figure 6C) behaved similarly, eluting with a K_{av} = 0.72; the calculated M_r (90×10^3) was ~40% larger than that for this activity from the low-KC extract.

The Ca^{2+}-independent activity in the high-KC extract eluted in two peaks with K_{av} = 0.36 and 0.81 corresponding to $M_r \sim 420 \times 10^3$ and 36×10^3, respectively (Figure 6C). This was substantially different than the profile for the same activity in the low-KC extract (Figure 6B). Since the extracts were loaded onto the column without prior adjustment of the KCl or CHAPS concentrations, it was possible that the low-KC activity peak with a K_{av} = 0.09 represented high MW aggregates that were dissociated in the presence of 1.5% CHAPS and 2.0 M KCl in the high-KC extract. If this were true, then if the CHAPS and the KCl concentrations in the low-KC extract were increased to 1.5% and 2.0 M, respectively, we should obtain a similar profile to that seen with the high-KC extracts. When this was tested, the Ca^{2+}-independent activity eluted in four peaks of K_{av} = 0.02, 0.30, 0.54, and 0.85 with calculated M_rs $\sim 1 \times 10^6$, 420×10^3, 125×10^3, and 36×10^3, respectively (data not shown). This result suggests that the Ca^{2+}-independent HA receptor aggregates to form high MW complexes, depending on the KCl and/or CHAPS concentrations.

The above results indicate that the Ca^{2+}-independent endocytic HA receptor is physically different and substantially larger than the Ca^{2+}-dependent HA-binding activity. The specific HA-binding activity of the Ca^{2+}-independent HA receptor in the low-KC peak fraction was ~56 fmol of HA/μg of protein. This represents a ~26-fold purification of the receptor relative to isolated LECs.

Discussion

The LEC endocytic HA receptor is a Ca^{2+}-independent class II receptor whose function is to remove HA and other GAGs from the circulation. These polysaccharides are internalized

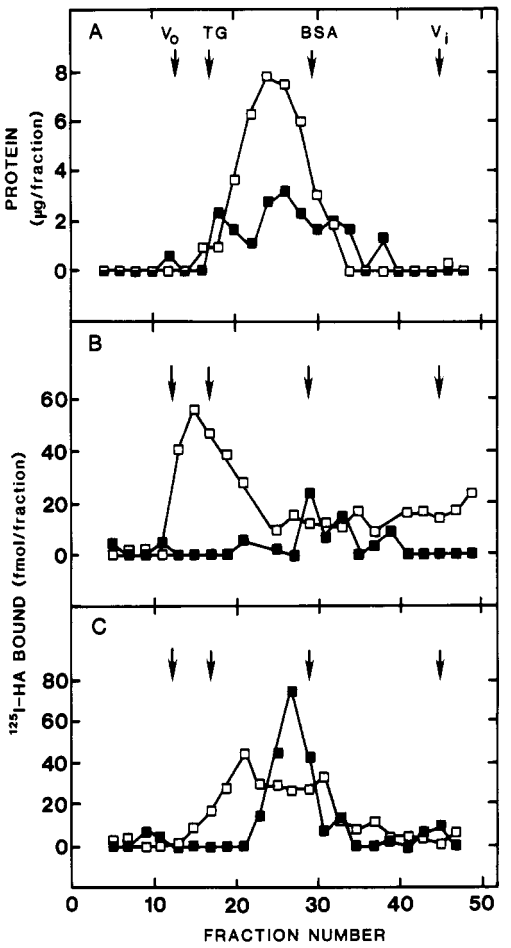

FIGURE 6: Separation of the Ca^{2+}-independent HA receptor and the Ca^{2+}-dependent HA-binding activity using gel filtration chromatography. Aliquots of the unadjusted low-KC and high-KC extracts (150 μL) were chromatographed separately on a Sephacryl S-400 column (0.75 × 14 cm) equilibrated in buffer 1 containing 0.03% CHAPS, 5 mM EDTA, and 2.0 M KCl (column buffer). The columns were eluted with the same buffer at a flow rate of 0.5 mL/min, and ~170-μL fractions were collected. In all experiments, the even-numbered fractions were assayed for protein content using the method of Bradford (1976) and the odd-numbered fractions were used to determine the Ca^{2+}-independent and Ca^{2+}-dependent specific ^{125}I-HA-binding activities using the dot blot assay as described for Figures 4 and 5. Arrows indicate the elution positions of blue dextran 2000 and phenol red, which were used to determine V_0 (~2.3 mL) and V_i (~5.4 mL), respectively, and BSA and thyroglobulin (TG), which were used to calibrate the column. Panel A: The protein elution profiles of the low-KC (□) and high-KC (■) extracts; the samples contained 200 and 100 μg of protein, respectively. Panel B: The elution profiles of the Ca^{2+}-independent (□) and the Ca^{2+}-dependent (■) ^{125}I-HA-binding activity in the low-KC extract. Panel C: The elution profiles of the Ca^{2+}-independent (□) and the Ca^{2+}-dependent (■) ^{125}I-HA-binding activity in the high-KC extract.

by receptor-mediated endocytosis via a coated pit pathway (McGary et al., 1989; Smedsrod et al., 1988, 1990) and delivered to lysosomes where they are degraded. The function, binding specificity, and affinity of this HA receptor have been characterized in isolated LECs [see Smedsrod et al. (1990) for review; Raja et al., 1988; McGary et al., 1989]. Although extensive studies have also been performed on the function of this important receptor in live mammals, including human (Smedsrod et al., 1990), little is known about its molecular properties or composition. To investigate its biochemical and physical properties, the receptor was solubilized using the detergent CHAPS under conditions that retained its specific cation-independent HA-binding activity as demonstrated using a dot blot assay. We also observed the simultaneous solubilization of a Ca^{2+}-dependent HA-binding activity from LEC membranes.

We have previously reported that a Ca^{2+}-dependent HA binding activity is associated with cultured LECs and that this activity is functionally distinct from the endocytic HA receptor (Yannariello-Brown et al., 1992). Here, we present evidence that both HA-binding activities are membrane-associated components and that these two activities are not only functionally distinct, but biochemically distinct as well. This conclusion is based on the following observations. (1) The two HA-binding activities require different concentrations of KCl and CHAPS for extraction from LEC membranes. (2) The two CHAPS-solubilized activities have different sensitivities to reduction. The Ca^{2+}-independent HA receptor was inactivated by reduction with DTT, while reduction had little effect on the Ca^{2+}-dependent activity. (3) The two HA-binding activities are affected very differently by temperature. Treatment at 56 °C inactivated the Ca^{2+}-independent HA receptor, whereas it stimulated the Ca^{2+}-dependent activity. (4) The two HA-binding activities could be separated from each other by gel filtration chromatography.

The Ca^{2+}-independent HA receptor in LECs has been shown to recycle (McGary et al., 1989). Consistent with an intracellular recycling itinerary for this receptor, it is found both on the cell surface and inside the cell (Raja et al., 1988); specific HA-binding activity can be readily measured on the cell surface, as well as internally after LECs are permeabilized with 0.055% digitonin. In contrast, the Ca^{2+}-dependent HA-binding activity was only detected extracellularly, presumably on the cell surface (Yannariello-Brown et al., 1992). That both activities were associated with the surface of LECs in culture and with isolated LEC membranes raised the possibility that they could be integral membrane components. The finding that both activities are resistant to release by mild alkaline treatment suggests that both activities either are integral membrane components or are associated with the cytoskeleton. This result was expected for the Ca^{2+}-independent endocytic HA receptor, since most, if not all, class II receptors that have been investigated to date are integral membrane proteins (Anderson & Kaplan, 1983).

Most cell surface membrane-bound receptors, including the other class II endocytic receptors, have multiple disulfide bonds in their external ligand binding domains. These disulfide bonds are very important for maintaining the structural integrity and ligand binding capability of these receptors (Geisow, 1989). There is a high level of conservation of the critical Cys residues required for the proper folding and organization of the ligand binding domains among members of the same receptor family (Drickamer, 1988). Since the Ca^{2+}-independent HA receptor is a class II endocytic receptor, it is, therefore, not surprising that treatment with a reducing agent destroyed its HA-binding activity. This observation may explain our failure to demonstrate ^{125}I-HA binding to LEC membrane extracts that had been transferred to nitrocellulose from reduced SDS–PAGE gels in a modified "Western blot". The finding that intact disulfide bonds are required for the HA-binding activity of the Ca^{2+}-independent HA receptor is also consistent with results from studies performed with other HA-binding molecules. Hardingham et al. (1976) demonstrated that reduction and alkylation of cartilage proteoglycan, but not alkylation alone, prevented its interaction with HA. Whether the sensitive disulfide bonds in the Ca^{2+}-independent receptor are within or between possible subunits of the receptor cannot be addressed at this point. Surprisingly, the Ca^{2+}-dependent HA-binding activity was insensitive to reduction and alkylation.

It is likely, therefore, that the structural features needed for its interaction with HA are not dependent on the integrity of disulfide bonds. These data argue against, but do not rule out, the possibility that the Ca^{2+}-dependent HA-binding activity is a proteoglycan-like molecule. Since neither HA-binding activity was inhibited by alkylation alone, it is also unlikely that either molecule requires a free sulfhydryl group in order to bind HA.

The sensitivity of the Ca^{2+}-independent HA receptor to inactivation by treatment at relatively low temperatures suggests the receptor is a protein. The temperature lability of the HA receptor contrasts sharply with the substantial apparent activation of the Ca^{2+}-dependent HA-binding activity with increasing temperature. Possible explanations for this increased activity could be that the increase in temperature induces conformational changes in the molecule to expose additional HA-binding sites (i.e., increases the valency) or inactivates a thermal-sensitive inhibitor of the Ca^{2+}-dependent HA-binding activity.

The selective extraction of the two HA-binding activities by CHAPS, using the two-cycle extraction protocol, strongly suggests that they possess important structural differences. About 50% of the Ca^{2+}-independent activity, with little Ca^{2+}-dependent activity, is extracted into the low-KC buffer, and the KCl and CHAPS concentrations must be increased substantially in order to extract the Ca^{2+}-dependent activity. This preferential extraction has allowed us to partially separate the two activities and enrich for the Ca^{2+}-independent HA receptor. When gel filtration chromatography was combined with selective solubilization, complete separation of the Ca^{2+}-independent and Ca^{2+}-dependent HA-binding activities was achieved. This result further supports the conclusion that the two HA-binding activities are mediated by distinct molecules, although we cannot rule out the possibility that they both share common sequences or structural motifs. Purification of these HA-binding molecules will allow us to address this issue.

The apparent sizes of the HA receptor and the Ca^{2+}-dependent HA-binding activity were clearly different as assesed by gel filtration. The Ca^{2+}-dependent activity was smaller [$M_r \sim (66–90) \times 10^3$] than most of the HA receptor activity. That multiple species of HA receptor were detected, depending on the concentrations of CHAPS and KCl, suggests that either the HA receptor forms oligomers or that limited proteolysis of the receptor occurred. The largest species of HA receptor corresponded to M_rs $\approx 10^6$ and 4.2×10^5. Since these apparent sizes include detergent, we cannot yet estimate the true size of the native receptor. However, we have recently succeeded in specifically cross-linking a unique HA photoaffinity probe to permeable LECs (Yannariello-Brown & Weigel, 1991). Two large proteins of M_r = 166 000 and 175 000 were equally labeled. Cross-linking to both proteins was >90% competed by a 100-fold excess of nonlabeled HA. If the functional LEC HA receptor is a heterooligomer of these two subunits, it would be ~340 000 and perhaps correspond, with associated CHAPS, to the peak observed at $M_r \sim 4.2 \times 10^5$. Studies are in progress to determine this.

As this work was being prepared for submission, Forsberg and Gustafson (1991) reported a 100-kDa protein on the surface of rat LECs that could be iodinated and solubilized with Triton X-100 and that bound to HA–Sepharose. This protein was very readily degraded and appeared to be composed of 2–4 polypeptides held together by disulfide bonds. However, since the 100-kDa protein was not always detected and the number and MW of the subunits were also not consistent between preparations, the subunit composition is uncertain. The relationship between this protein and the LEC HA receptor characterized in the present study must now be elucidated.

Acknowledgments

We thank Paige Smith and Shirley Chapman for performing the liver perfusions and for help in preparing the membranes. We thank Lisa Raney and Becky Hansen for help in preparing the manuscript and Janet Oka for preparing the figures. We also thank the other members of our laboratory, both past and present, for their helpful discussions and interesting suggestions.

Registry No. HA, 9004-61-9; CHAPS, 75621-03-3; Ca, 7440-70-2; KCl, 7447-40-7.

References

Anderson, R. G. W., & Kaplan, J. (1983) *Mod. Cell Biol. 1*, 1–52.

Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., & Seed, B. (1990) *Cell 61*, 1303–1313.

Balazs, E. A., Watson, D., Duff, I. F., & Roseman, S. (1967) *Arthritis Rheum. 10*, 357–376.

Bertolami, C. N. (1984) *Soft and Hard Tissue Repair*, pp 67–97, Praeger Scientific, New York.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brecht, M., Mayer, U., Schlosser, E. and Prehm, P. (1986) *Biochem. J. 239*, 445–450.

Delpech, B., Vidard, M.-N., & Delpech, A. (1976) *Immunochemistry 13*, 111–116.

Drickamer, K. (1988) *J. Biol. Chem. 263*, 9557–9560.

D'Souza, M. D., & Datta, K. (1985) *Biochem. Int. 10*, 43–51.

Eriksson, S., Fraser, J. R. E., Laurent, T. C., Pertoft, H., & Smedsrod, B. (1983) *Exp. Cell. Res. 144*, 223–228.

Evered, D., & Whelan, J. (1989) *The Biology of Hyaluronan*, Wiley, Chicester, U.K.

Forrester, J. V., & Wilkinson, P. C. (1981) *J. Cell Sci. 48*, 315–331.

Forsberg, N., & Gustafson, S. (1991) *Biochim. Biophys. Acta 1078*, 12–18.

Fraser, J. R. E., & Laurent, T. C. (1989) in *The Biology of Hyaluronan* (Evered, D., & Whelan, J., Eds.) pp 41–59, Wiley, Chicester, U.K.

Fraser, J. R. E., Kimpton, W. G., Laurent, T. C., Cahill, R. N. P., & Vakakis, N. (1988) *Biochem. J. 256*, 153–158.

Frost, S. J., & Weigel, P. H. (1990) *Biochim. Biophys. Acta. 1034*, 39–45.

Frost, S. J., McGary, C. T., Raja, R. H., & Weigel, P. H. (1988) *Biochim. Biophys. Acta 946*, 66–74.

Frost, S. J., Raja, R. H., & Weigel, P. H. (1990) *Biochemistry 29*, 10425–10432.

Fujiki, Y., Hubbard, A. L., Fowler, S., & Lazarow, P. B. (1982) *J. Cell Biol. 93*, 97–102.

Geisow, M. J. (1989) *Bio Essays 4*, 149–151.

Green, S. J., Tarone, G., & Underhill, C. B. (1988) *Exp. Cell Res. 178*, 224–232.

Gustafson, S., & Forsberg, N. (1991) *Biochim. Biophys. Acta 1091*, 36–40.

Hardingham, T. E., Ewins, R. J. F., & Muir, H. (1976) *Biochem. J. 157*, 127–143.

Hascall, V. C. (1977) *J. Supramol. Struct. 7*, 101–120.

Hascall, V. C., & Hascall, G. K. (1981) in *Cell Biology of the Extracellular Matrix*, pp 39–63, Plenum Press, New York.

Hutadilok, N., Ghosh, P., & Brooks, P. M. (1988) *Ann. Rheum. Dis. 47*, 377–385.

Knudson, W., Biswas, C., Li, X.-Q., Nemec, R. E., & Toole, B. P. (1989) in *The Biology of Hyaluronan* (Evered, D., & Whelan, J., Eds.) pp 150-169, Wiley, Chichester, U.K.
LeBoeuf, R. D., Raja, R. H., Fuller, G. M., & Weigel, P. H. (1986) *J. Biol. Chem. 261*, 12586-12592.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
McGary, C. T., Raja, R. H., & Weigel, P. H. (1989) *Biochem. J. 257*, 875-884.
Oka, J. A., & Weigel, P. H. (1987) *J. Cell Physiol. 133*, 243-252.
Perides, G., Lane, W. S., Andrews, D., Dahl, D., & Bignami, A. (1989) *J. Biol. Chem. 264*, 5981-5987.
Raja, R. H., LeBoeuf, R. D., Stone, G. W., & Weigel, P. H. (1984) *Anal. Biochem. 139*, 168-177.
Raja, R. H., McGary, C. T., & Weigel, P. H. (1988) *J. Biol. Chem. 263*, 16661-16668.
Scott, J. E. (1960) *Methods Biochem. Anal. 8*, 145-197.
Smedsrod, B., Pertoft, H., Eriksson, S., Fraser, J. R. E., & Laurent, T. C. (1984) *Biochem. J. 223*, 617-626.
Smedsrod, B., Malmgren, M., Ericsson, J., & Laurent, T. C. (1988) *Cell Tissue Res. 235*, 39-45.
Smedsrod, B., Pertoft, H., Gustafson, S., & Laurent, T. C. (1990) *Biochem. J. 266*, 313-327.
Smith, C. E., Musich, P. R., & Johnson, D. A. (1989) *Anal. Biochem. 177*, 212-219.
Stamenkovic, I., Aruffo, A., Amiot, M., & Seed, B. (1991) *EMBO J. 10*, 343-348.
Toole, B. (1981) in *Cell Biology of the Extracellular Matrix* (Hay, E. D., Ed.) pp 259-294, Plenum Press, New York.
Turley, E. A. (1984) *Cancer Metastasis Rev. 3*, 325-339.
Turley, E. A., Brassel, P., & Moore, D. (1990) *Exp. Cell Res. 187*, 243-249.
Underhill, C. B., & Dorfman, A. (1978) *Exp. Cell Res. 117*, 155-164.
Underhill, C. B., & Toole, B. P. (1980) *J. Biol. Chem. 255*, 4544-4549.
Weigel, P. H., Fuller, G. M., & LeBoeuf, R. D. (1986) *J. Theor. Biol. 119*, 219-234.
Yannariello-Brown, J., & Weigel, P. H. (1991) *J. Cell Biol. 115*, 262 (Abstract).
Yannariello-Brown, J., Wewer, U., Liotta, L. A., & Madri, J. A. (1988) *J. Cell Biol. 106*, 1773-1786.
Yannariello-Brown, J., McGary C. T., & Weigel, P. H. (1992) *J. Cell. Biochem.* (in press).
Yoneda, M., Suzuki, S., & Kimata, K. (1990) *J. Biol. Chem. 265*, 5247-5257.

Hapten Conformation in the Combining Site of Antibodies That Bind Phenylphosphocholine†

Urs Bruderer,*,‡,§ David H. Peyton,*,∥ Elisar Barbar,∥ Jack H. Fellman,⊥ and Marvin B. Rittenberg‡
Department of Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201, Department of Chemistry, Portland State University, Portland, Oregon 97207-0751, and Department of Biochemistry, Oregon Health Sciences University, Portland, Oregon 97201

Received August 26, 1991; Revised Manuscript Received October 7, 1991

ABSTRACT: We have shown previously that anti-phenylphosphocholine antibodies elicited by phosphocholine–keyhole limpet hemocyanin can be divided into two populations according to their ability to recognize the two hapten analogues *p*-nitrophenylphosphocholine (NPPC) and *p*-nitrophenyl 3,3-dimethylbutyl phosphate (NPDBP). These analogues differ from each other in that NPPC has a positively charged nitrogen in the choline moiety, whereas NPDBP lacks the positively charged nitrogen. Group II-A antibodies bind only NPPC, whereas group II-B antibodies bind both ligands. Here, by infrared and nuclear magnetic resonance spectroscopic investigations, we find that when free in solution NPPC has a predominantly fixed structure in which the termini approach each other, probably due to electrostatic interactions within the molecule; this "bent" structural feature is retained when the ligand is bound by antibody. In contrast, the structure of unbound NPDBP is less fixed, being characterized by rapidly interchanging conformations corresponding to an open chain structure with less overall proximity of the termini compared to NPPC. The overall shape of NPPC is essentially unaltered by binding, whereas in the case of NPDBP what was a minor conformation in the unbound state becomes the predominate conformation of the bound ligand. Thus, our results are consistent with these antibodies providing a molecular template for stabilizing the conformation of the bound ligand.

The majority of antibodies elicited by phosphocholine–keyhole limpet hemocyanin (PC–KLH)[1] belong to two populations which differ in their fine specificities. Group I antibodies react predominantly with the phosphocholine (PC) moiety, whereas group II antibodies require a phenyl group and have affinity for molecules such as *p*-nitrophenylphosphocholine (NPPC);

† This work was supported in part by a grant from the Oregon Affiliate of the American Heart Association to D.H.P. and NIH Grants AI14985 and AI26827 to M.B.R.
* Corresponding authors.
‡ Department of Microbiology and Immunology, Oregon Health Sciences University.
§ Present address: Department of Immunology, Swiss Serum and Vaccine Institute, CH-3001 Berne, Switzerland.
∥ Department of Chemistry, Portland State University.
⊥ Department of Biochemistry, Oregon Health Sciences University.

[1] Abbreviations: IR, infrared; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; NPDBP, *p*-nitrophenyl 3,3-dimethylbutyl phosphate; NPPC, *p*-nitrophenylphosphocholine; PC–KLH, phosphocholine–keyhole limpet hemocyanin; TRNOE, transferred nuclear Overhauser effect.

0006-2960/92/0431-584$03.00/0