Hyaluronan (HA) and chondroitin sulfate clearance from lymph and blood is mediated by the hyaluronan receptor for endocytosis (HARE). The purification and molecular cloning (Zhou, B., Weigel, J. A., Saxena, A., and Weigel, P. H. (2002) Mol. Biol. Cell 13, 2853–2868) of this cell surface receptor were finally achieved after we developed monoclonal antibodies (mAbs) against HARE. There are actually two independent isoreceptors for HA, which in rat are designated the 175-kDa HARE and 300-kDa HARE. Only one mAb (number 174) effectively and completely blocked the specific uptake of $^{125}$I-HA at $37^\circ$C by rat liver sinusoidal endothelial cells. $^{125}$I-HA binding to both the 175-kDa and 300-kDa HARE proteins in a ligand blot assay was almost completely inhibited by $<1\mu g/ml$ mAb-174, whereas mouse IgG had little or no effect. MAb-174 also performed very well in Western analysis, indirect fluorescence microscopy, and a variety of immuno-procedures. Immunohistochemistry using mAb-174 localized HARE to the sinusoidal cells of rat liver, spleen, and lymph node. Western analysis using mAb-174 revealed that the sizes of both HARE glycoproteins were the same in these three tissues. $^{125}$I-HA was taken up and degraded by excised rat livers that were continuously perfused with recirculating medium. This HA clearance and metabolism by liver, which is a physiological function of HARE, was very effectively blocked by mAb-174 but not by mouse IgG. The results indicate that mAb-174 will be a useful tool to study the functions of HARE and the physiological significance of HA clearance.

After Meyer and Palmer (1) discovered hyaluronan (HA), it was found to be a component of essentially all vertebrate extracellular matrices (ECMs). Fibroblasts, keratinocytes, chondrocytes, and other cells continuously synthesize and secrete HA, which is a linear polymer with a native molecular mass that may exceed $10^6$ Da and is composed of the repeating disaccharide 2-deoxy-2-acetamido-2-glucopyranosyl-$\beta$ (1, 4)-2-acetamido-2-glucopyranosyl-$\beta$ (1, 3). Despite its simple structure, HA is involved in many cell functions including migration, differentiation, and phagocytosis (2–6). HA is important in development (4, 7), wound healing (8, 9), angiogenesis (10, 11), and tumor growth and metastasis (12, 13). Although previously believed to be only a structural component in the ECM, HA is now also recognized as an active cell-signaling molecule. Some cell types show distinct physiological responses to HA of different sizes. In particular, some cell types respond physiologically to very small, but not large, HA. Small HA oligosaccharides containing 14–20 sugars stimulate angiogenesis by endothelial cells (10, 11, 14), induce gene expression in activated macrophages (15), and induce NO synthase expression in sinusoidal LECs and Kupffer cells, but not hepatocytes or stellate cells (16).

Cell surface HA receptors identified to date include CD44, RHAMM (CD168), ICAM-1 (CD54), LYVE-1 (5), and an endocytic receptor that is specific for HA and chondroitin sulfate. This latter HA receptor is expressed in LECs, which remove HA and chondroitin sulfate from the blood (2, 17–20). Because this endocytic receptor is also present in other tissues and is not a liver-specific HA receptor, it was renamed HARE, the HA receptor for endocytosis (21). Unlike the other cell surface receptors for HA, HARE mediates the rapid endocytosis of HA and chondroitin sulfate via the clathrin-coated pit pathway (19, 22). HARE is, therefore, similar in its mode of action to the transferrin, asialoglycoprotein, mannose, and low density lipoprotein receptors (23).

After the discovery that liver is responsible for HA clearance from the blood (reviewed in Ref. 2), Deaciuc et al. (24, 25) demonstrated the ability of isolated perfused rat liver to take up HA. This uptake process was saturable at an HA concentration of $<0.15\mu g/ml$ with a steady-state uptake rate of $<10\mu g$ HA/h/g wet weight of liver. Deaciuc et al. (24, 25) also showed that galactosamine-induced hepatitis in rats was associated with elevated plasma HA levels and decreased HA clearance by perfused liver. These and similar observations by other investigators led to the realization that the HA clearance ability of liver can indicate the functional status of LECs and, thus, the general health of the liver. For example, Itasaka et al. (26) and Rao et al. (27) found that the HA clearance function was a useful parameter for predicting the likely success of human liver transplantation.

We previously identified two large membrane proteins of $\sim 175$ kDa and $\sim 300$ kDa in rat LECs that were specifically labeled with a photoaffinity derivative of HA (28) and retained specific HA binding activity in a novel ligand blot assay following SDS-PAGE and renaturation (29). The development of specific mAbs raised against the 175-kDa protein enabled these two HARE proteins to be purified from isolated rat LECs (30) and more recently from human spleen (31). After cloning the cDNA for the rat 175HARE, we stably expressed the recombi-
niant protein in SK-HeP-1 cells and found that this smaller HARE species can function as an endocytic recycling receptor with specificity for HA and chondroitin sulfate. The native rat 175HARE is derived by proteolysis from a larger precursor protein (32), probably the largest subunit of the 300HARE. The evidence so far indicates that the 175- and 300-kDa proteins are closely related but functionally independent HARE species. As previously described (21), all of the mAbs generated against rat HARE, designated mAb-174, inhibits 125I-HARE endocytosis by isolated rat LECs and inhibits 125I-HA binding to both HARE species in a ligand blot assay (21). This Ab was also able to immunoprecipitate HARE from isolated LECs. Here we report that mAb-174 also recognizes both HARE species from whole spleen, lymph node, and liver in Western analysis and performs well in immunolocalization and confocal microscopy procedures. More notably, mAb-174 also specifically blocked the uptake and degradation of 125I-HA by isolated perfused liver. This antibody should, therefore, be a valuable reagent in future studies of HARE function.

EXPERIMENTAL PROCEDURES

Materials and Media—125I-HA was prepared as described (33) using a hexamethylene derivative of HA (oligosaccharides; Mr, ~70,000). Male Sprague-Dawley rats (200 g) were from Charles River Labs. BSA fraction V was from Intergen. Hanks’ balanced salt solution and PBS were formulated according to the Invitrogen catalog formulations. Medium 1 was Eagle’s basal medium (Invitrogen no. 41500-018) supplemented with 100 mg/ml L-glutamine, 75 mg/ml sodium pyruvate, 2.4 mg/ml HEPES, and 0.22%/liter NaHCO3. Medium 1BSA was Medium 1 supplemented with 0.1% BSA (w/v). Collagenase was from Roche Molecular Biochemicals. The preparation of mouse mAbs against the rat HARE was described (21). Tris, SDS, ammonium persulfate, N,N,N′-methylenebisacrylamide, and SDS-PAGE standards were from Bio-Rad. Rat lymph nodes were a special purchase from Pel-Freeze Biologicals. Unless noted otherwise, other chemicals and reagents were from Sigma.

Isolation and Culture of LECs—Rat livers were perfused with collagenase using a modification of the procedure developed by Seglen (34) as described previously (35). LECs were isolated using differential centrifugation and discontinuous Percoll gradients (36). LECs banding at the 25/50% interface were removed, washed twice with RPMI 1640 (Invitrogen) containing penicillin/streptomycin (100 units/ml) each and 2 mM glutamine and suspended at 1.5–2 × 106 cells/ml. The cells were incubated first on a glass Petri dish for 10 min at room temperature to remove Kupffer cells, and then plated on human fibronectin-coated (50 μg/ml) 24-well tissue culture plates for endocytosis experiments or on glass coverslips for microscopy. After incubation at 37 °C for 2 h in a 5% CO2 atmosphere, the cells were washed three times with PBS, once with RPMI 1640, and then incubated in RPMI 1640 without serum at 37 °C for 1 h if they were to be used immediately or with 2% heat-inactivated bovine serum if they were to be cultured overnight before use. The 37 °C pretreatment was performed in all experiments to allow any endogenous cell surface-bound HA, e.g. from the serum, to be removed either by internalization or dissociation and washing.

Clearance of 125I-HA by Perfused Liver—Rat livers were excised following a standard perfusion protocol (35). During excision and mounting in a recirculation apparatus, they were perfused without recirculation with Buffer 1 (142 mM NaCl, 6.7 mM KCl, and 10 mM HEPES, pH 7.4) for 8–10 min at 35 °C. Washed livers were then perfused for 15 min with recirculation with a recirculation medium containing HEPES, pH 7.4, NaHCO3, and Medium 1BSA. After flushing with Buffer 1, the liver was allowed to take up HA for 10 min at 35 °C. Samples (300 μl) of the perfusate were removed and divided into 50-μl portions for determination of total radioactivity (in duplicate) or HA degradation products (in triplicate). Competitor HA (50 μg/ml) or mouse IgG or mAb-174 IgG (5 μg/ml) was added to the recirculation medium containing 175HARE and mixed well before the recirculating perfusate was started. The first sample taken immediately after starting the perfusion was used to determine the starting values, because there was a substantial (~15%) and reproducible dilution of the recirculating medium by the residual buffer in the liver. In experiments with purified IgGs, these were also included during the 35 °C pre-perfusion treatment at the same concentration used in the experiment.

Degradation of 125I-HA by Perfused Liver—Degradation of 125I-HA was measured by a cetylpyridinium chloride precipitation assay as described previously (20). Samples (50 μl in triplicate) of recirculation medium containing 125I-HA were added to 250 μl of 1 mg/ml HA in 1.5-ml microfuge tubes. After mixing, 300 μl of 6% (w/v) cetylpyridinium chloride in distilled water was added, and the tubes were mixed by vortexing. After 10 min at room temperature, the samples were centrifuged at 9000 rpm in an Eppendorf model 5417 microcentrifuge at room temperature for 5 min. A sample (300 μl) of the supernatant was taken for determination of radioactivity, and the remainder was removed by aspiration. The tip of the tube containing the precipitate pellet was cut off, put in a γ counter tube, and radioactivity was determined. Degradation was measured as the time-dependent increase of non-precipitable radioactivity. >80% of the total radioactivity was precipitable by the beginning of the experiment.

Immunocytochemistry—Lymph node, liver, and spleen tissues from Sprague-Dawley rats were removed, fixed in 10% neutral buffered formalin, processed, and paraffin embedded overnight on a Tissue Tek VIP processor. Tissue sections (5 μm) were collected on charged slides and dried at 60 °C overnight. The slides were dewaxed three times for 3 min each with xylene followed by four washes for 3 min each with alcohol (100%, 95%, 90%, then 70%) and a single 2-min wash in water at room temperature. The endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide for 6 min followed by two 2-min water washes. The tissue sections on the slides were digested for 15 min at 37 °C in pre-warmed 0.1x HCl containing 0.32 mg/ml pepsin followed by a 2-min water wash and a 2-min PBS wash. The slides were washed with PBS and incubated with the appropriate primary antibody (~1:500) at room temperature for 60 min. After a 1 min PBS wash, the slides were treated with biotinylated horse anti-mouse IgG (1:200) for 30 min at room temperature. After another PBS rinse, the slides were incubated with streptavidin-horseradish peroxidase (1:100, Jackson Labs) for 30 min, washed once with PBS, and once with distilled water. Color development was for 5 min with 2.0% (v/v) aminoethylcarbazine and hydrogen peroxide (ScyTek Laboratories, Logan Utah) followed by counterstaining with hematoxylin. Slides were viewed with an Olympus DP70 light microscope equipped with an Olympus DP10 digital camera.

RESULTS

To purify and characterize the rat HARE proteins, we developed a panel of eight mAbs raised against the partially purified 175HARE (21, 30). Surprisingly, all of these mAbs also recognized the 300HARE in either Western or immunopurification assays. We now know that this is because the single 175HARE species and probably the 230-kDa and 250-kDa subunits of the 300HARE are derived by proteolysis from a larger precursor (32). Fig. 1 shows the concentration-dependent blocking of specific uptake of 125I-HA by LECs at 37 °C. Specific internalization was completely inhibited by 5 μg/ml mAb-174, whereas up to 10 μg/ml of three other anti-HARE mAbs inhibited only ~15%. Interestingly, mAb-235 reproducibly inhibited 125I-HA endocytosis by LECs by about 50%. No further inhibition by the other six mAbs (only numbers 28, 30, and 467 are shown) or mouse IgG (not shown) occurred regardless of IgG concentration or duration of treatment.

Previously, we reported that mAb-174 blocks 125I-HA binding to both HARE species (21) in a ligand blot assay that detects the activity of these proteins after SDS-PAGE, electrophoresis, and renaturation (29). In dose-response experiments, <1 μg/ml mAb-174 blocked 125I-HA binding to both the affinity-purified 175HARE and 300HARE proteins, but control IgG up to 10 μg/ml had no effect (not shown). Inhibition of binding to the 175HARE was almost complete; ~88% of the specific binding was blocked at 2–10 μg/ml mAb-174. Although the effect of mAb-174 on 125I-HA binding to the 300HARE was identical to that of the 175HARE below 1 μg/ml, inhibition
leveled off at ~50% between 1–10 μg/ml.

Despite the ability of mAb-174 to inhibit HA uptake by LECs at 37 °C or HA binding in the ligand blot assay at room temperature, we unexpectedly found that mAb-174 did not block 125I-HA binding to LECs at 4 °C (Fig. 2). To test the possibility that this inability to inhibit HA binding was due to an inherent difference in HARE between 37 °C and 4 °C (e.g. a conformation change), we fixed LECs so that endocytosis could not occur when the cells were subsequently put at 37 °C. This enabled us to determine whether mAb-174 could block 125I-HA binding per se at 37 °C; this cannot be assessed if endocytosis occurs simultaneously. The results confirmed that mAb-174, but none of the other HARE-specific Abs tested, could block 125I-HA binding to fixed LECs at 37 °C. We conclude that HARE likely undergoes a conformation change between 37 °C and 4 °C that does not prevent 125I-HA binding to LECs but that alters the epitope recognized by mAb-174 so that it does not block HA binding.

We found previously that mAb-174 recognizes the 175HARE and 300HARE proteins in Western analysis of crude LEC membranes (21). Because we had not tested any of our mAbs individually using other tissues, we compared the Western blot reactivity of mAb-174 with the two HARE species from rat lymph node, spleen, and liver. In all three tissues, the HARE proteins were visualized by mAb-174 (Fig. 3). Expression of the 175HARE in lymph node was lower than in the other tissues, although it was detectable (not shown). In addition to its utility as a ligand-blocking antibody and for Western analysis, mAb-174 also worked well in a variety of localization procedures, including indirect fluorescence microscopy (e.g. the same localization pattern was observed previously with a mixture of mAbs; Ref. 21) and immunohistochemistry (Fig. 4). Immunohistochemical localization of HARE using mAb-174 revealed heavy staining of the sinusoidal endothelial cells in liver, spleen, and lymph node (Fig. 4). The medullary sinuses of lymph node and the venous sinusoids of spleen contain large amounts of HARE protein.

The fact that mAb-174 effectively inhibits HA binding and uptake by isolated LECs at 37 °C does not necessarily mean that the antibody would be equally able to inhibit HA clearance by these cells in intact liver. To answer this question, we tested the ability of mAb-174 to block 125I-HA uptake and degradation by excised rat livers that were continuously perfused ex vivo. In these experiments we used a concentration of mAb-174 (5 μg/ml) that gave essentially complete inhibition of uptake by LECs. 125I-HA introduced into the perfusion recirculating medium was readily removed; ~50% was cleared within ~20 min (Fig. 5). In the presence of excess unlabeled HA, the removal of 125I-HA was completely blocked. Although control mouse IgG appeared to retard slightly the clearance of 125I-HA, there was no statistically significant difference between most of the data pairs. In contrast, when mAb-174 was present there were very significant differences in 125I-HA removal at all times during the perfusion, and the level of residual uptake was just slightly greater than that in
the presence of excess unlabeled HA. The results confirm that mAb-174 effectively blocks $^{125}$I-HA uptake by LECs in intact perfused liver.

This conclusion was further confirmed by demonstrating the effect of mAb-174 on the appearance of $^{125}$I-HA degradation products in the perfusion medium (Fig. 6). $^{125}$I-HA removal and processing by perfused liver was so efficient that degradation products were detected within minutes. By 30 min, about 25% of the total $^{125}$I-HA in the perfusate had been internalized, degraded, and released back into the medium. Again, no sig-

ificant differences were seen in the appearance of degradation products when mouse IgG was present, although all of the values were slightly lower. In contrast, mAb-174 substantially reduced the steady-state rate at which $^{125}$I-HA degradation products were released. The inhibition by unlabeled HA was essentially complete, indicating that >95% of the observed degradation was mediated by a specific mechanism.

**DISCUSSION**

HA is used in many cosmetic and clinical applications, and new HA-containing materials are currently being developed for medical use. For example, HA is used in ophthalmological surgery (40), to treat patients with osteoarthritis in knee and hip joints (41), to prevent adhesions after surgery (42), and as an aerosol to prevent elastase-mediated injury in pulmonary emphysema (43). Because of the wide range of medical uses for HA, it is very important to understand how HA turnover and clearance are regulated. The present study was undertaken as an initial effort to define the role of HARE in this process.

After LECs were found to contain an endocytic HA receptor (23, 44), others demonstrated that isolated perfused rat liver can remove circulating HA (24, 25, 45), presumably mediated by this HA receptor. The present results verify that this assumption was correct. The quantitative inhibition of $^{125}$I-HA uptake and degradation by mAb-174 demonstrates that HARE mediates at least 90% of the liver’s clearance ability. In this study, we did not perform an extensive dose response analysis because of the larger number of animals and amount of purified IgG required. Accordingly, we did not necessarily use an optimum dose of mAb-174 for blocking HARE in perfused liver, although the mAb-174 concentration used, 5 $\mu$g/ml, gave maximum inhibition of $^{125}$I-HA internalization by LECs (Fig. 1).

Using a mixture of anti-HARE mAbs, we found that the small and large rat HARE proteins are highly expressed in the sinusoids of liver, the venous sinuses of the red pulp in spleen, and the medullary sinuses in lymph nodes (21). This distribution was also seen using only mAb-174 (Fig. 4). HARE was not detectable by Western or immuno-cytochemical analysis in brain, lung, heart, muscle, kidney, or intestine. Abundant expression of HARE in the sinusoids of liver and lymphatic tissues is ideal for keeping the systemic HA levels low. Banerji et al. (46) discovered a lymph-specific homologue of CD44, designated LYVE-1, which binds HA and is localized to the luminal
Characteristics of a mAb That Blocks HARE Function

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A Blocking Antibody to the Hyaluronan Receptor for Endocytosis (HARE) Inhibits Hyaluronan Clearance by Perfused Liver
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