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Endocytosis of hyaluronic acid by rat liver endothelial cells

Evidence for receptor recycling

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Hyaluronic acid (HA) is cleared from the blood by liver endothelial cells through receptor-mediated endocytosis [Eriksson, Fraser, Laurent, Pertoft & Smedsrod (1983) Exp. Cell Res. **144**, 223–238]. We have measured the capacity of cultured rat liver endothelial cells to endocytose and degrade ^{125}I-HA ($M_r \sim 44000$) at 37 °C. Endocytosis was linear for 3 h and then reached a plateau. The rate of endocytosis was concentration-dependent and reached a maximum of 250 molecules/s per cell. Endocytosis of ^{125}I-HA was inhibited more than 92% by a 150-fold excess of non-radiolabelled HA. HA, chondroitin sulphate and heparin effectively competed for endocytosis of ^{125}I-HA, whereas glucuronic acid, *N*-acetylglucosamine, DNA, RNA, polygalacturonic acid and dextran did not compete. In the absence of cycloheximide, endothelial cells processed 13 times more ^{125}I-HA in 6 h than their total (cell-surface and intracellular) specific HA-binding capacity. This result was not due to degradation and rapid replacement of receptors, because, even in the presence of cycloheximide, these cells processed 6 times more HA than their total receptor content in 6 h. Also, in the presence of cycloheximide, no decrease in ^{125}I-HA-binding capacity was seen in cells processing or not processing HA for 6 h, indicating that receptors are not degraded after the endocytosis of HA. During endocytosis of HA at 37 °C, at least 65% of the intracellular HA receptors became occupied with HA within 30 min. This indicates that the intracellular HA receptors (75% of the total) function during continuous endocytosis. Hyperosmolarity inhibits endocytosis and receptor recycling in the asialoglycoprotein and low-density-lipoprotein receptor systems by disrupting the coated-pit pathway [Heuser & Anderson (1987) J. Cell Biol. **105**, 230a; Oka & Weigel (1988) J. Cell. Biochem. **36**, 169–183]. Hyperosmolarity inhibited ^{125}I-HA endocytosis in liver endothelial cells by more than 90%, suggesting use of a coated-pit pathway by this HA receptor. We conclude that liver endothelial cell HA receptors are recycled during the continuous endocytosis and processing of HA.

INTRODUCTION

Hyaluronic acid (HA), a glycosaminoglycan, is a ubiquitous component of the mammalian extracellular matrix and has been shown to have an important function in many cellular processes. Roles for HA have been shown in angiogenesis [1], tumour formation and metastasis [2,3], development and differentiation [4] and in several functions of the immune and inflammatory processes [5–9]. HA has been used clinically as a therapeutic agent for ophthalmic surgery [10], arthritis [11,12], tendon repair [13], and the prevention of contracture formation [14]. The therapeutic use of HA has led to an interest in the fate and metabolism of exogenously administered HA. Radiolabelled HA injected into the circulation of rabbits or rats is removed with a half-time of 2.5–4.5 min, and about 90% of the radiolabel is localized in the liver [15,16]. Separation and analysis of the various liver-cell types demonstrated that liver endothelial cells (LEC) are responsible for the internalization and degradation of HA [17–19]. A single class of high-affinity receptors for HA has been demonstrated on LEC [20,21]. The HA receptor on LEC appears to be different in both its function and sensitivity to pH [21] from the fibroblast HA receptor identified by Underhill & Toole [22].

The rapid plasma clearance of HA is mediated by LEC through a receptor-mediated endocytic process. However, the fate of the HA receptor after endocytosis is unknown. There are four possible outcomes for processing of endocytosed receptor–ligand complexes [23]. The receptor may recycle back to the cell surface while the ligand is degraded, as seen with the low-density-lipoprotein [24] or asialoglycoprotein [25] receptor systems. The receptor and ligand may both recycle, as seen with the transferrin receptor [26]. A third fate, for example seen with the epidermal-growth-factor receptor complex [27], is that both the receptor and the ligand can be degraded. Finally, both receptor and ligand may be transported across a polar cell and released into a different external compartment, as occurs during the transcytosis of immunoglobulin A receptor by secretory component [28]. Since LEC degrade HA after its internalization [17], only two of these four possible routes need to be considered. During the continuous endocytosis of HA, receptors could either be degraded or recycled.

In the present study we examined the endocytosis and degradation of ^{125}I-HA by LEC under a variety of

Abbreviations used: HA, hyaluronic acid; LEC, liver endothelial cells; CPC, cetylpyridinium chloride; PBS, phosphate-buffered saline (full composition and pH are given in the text); BSA, bovine serum albumin.

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conditions. The results indicate that LEC endocytose HA primarily by a receptor-mediated process that recognizes at least three types of glycosaminoglycans. Results of several types of experiments indicate that LEC HA receptors recycle during continuous endocytosis. The results also suggest that the intracellular receptors function during continuous endocytosis and that continuous endocytosis utilizes a coated-pit pathway. A preliminary report of these results has already been presented [29].

EXPERIMENTAL

Materials

HA (human umbilical cord), from Sigma, was further purified using cetylpyridinium chloride (CPC) fractionation on celite followed by ethanol precipitation as described by Scott [30]. The purified HA contained less than 1% protein and less than 0.1% sulphate by weight. Most of these studies employed a single batch of HA that was relatively small ($M_r \sim 44000$) and was not further fragmented. Where indicated in the Figure legends, some experiments were conducted using smaller HA ($M_r \sim 30000$). These latter oligosaccharides were generated by sonication of native HA under controlled conditions. A 10 ml portion of a 5 mg/ml solution of native HA in phosphate-buffered saline (PBS) was transferred to a 50 ml plastic tube in an ice bath. The sonicating probe was immersed in the HA solution to a level just above the bottom of the tube and sonication was at 70 W for 280 s using a Bronson CL 40002A sonicator. The HA oligosaccharides obtained were acid-hydrolysed under mild conditions (0.1 M-HCl, 2.5 h, 55 °C), neutralized and then fractionated over a Bio-Gel P-10 column. More than 90% of the loaded sugar was recovered in the void volume, indicating that very few oligosaccharides of less than 80 saccharide units were generated under these conditions. Oligosaccharides were converted into the HA–hexylamine, the HA–Bolton–Hunter adduct, and radioiodinated as previously described [31], with the following minor modification. To remove charged diaminohexane from the HA–hexylamine, the reaction mix was adjusted to pH 2.5 with acetic acid and ethanol was added to precipitate HA–amine [31]. The pellet was dissolved in distilled water, the pH was adjusted to 11 with NaOH and then the HA–hexylamine was ethanol-precipitated again. The procedure of an acidic-ethanol precipitation followed by a basic-ethanol precipitation was repeated two or three times until the HA–hexylamine was purified from the free amine.

1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (Iodogen) was from Pierce, and Sephadex G-15 was from Pharmacia. Percoll, cycloheximide, chondroitin sulphate (mixed isomers), *NN'*-diacetylchitobiose, glucuronic acid, *N*-acetylglucosamine, polygalacturonic acid and inulin were from Sigma. BSA (fraction V) was from either Sigma or Armour Pharmaceutical. Digitonin was from either Sigma or Kodak. Other investigators should note that the purity of digitonin from Sigma has decreased, so that 1.4% stock solution in ethanol [32] can no longer be prepared. We find that Kodak presently supplies the most suitable digitonin for the use described here. $Na^{125}I$ (10–20 mCi/μg of iodine) was from Amersham International, and NET-250 ^3H-labelled L-amino acid mixture was from New England Nuclear. De-sulphated chondroitin sulphate was a gift from Mr. Stephen Frost (this Department) and was prepared by the procedure of Nagasawa & Inoue [33]. Dextran and dextran sulphate were from Pharmacia, and heparin was from V Labs. Bio-Gel P-2 gel-filtration medium (200–400 mesh) was from Bio-Rad. Coomassie Blue G-250 protein assay reagent was from Pierce, and bisbenzimide (Hoeschst dye 33258) was from Behring Diagnostics.

Research-grade collagenase from *Clostridium histolyticum* was from Serva. Male Sprague–Dawley rats (150–350 g) were from Harlan Breeding Laboratories, Houston, TX, U.S.A. RPMI 1640 culture medium, as a powder, was from Gibco and was prepared as described by the manufacturer. Complete medium also contained penicillin/streptomycin (Gibco) to a final concentration of 100 units/ml (each), gentamycin (Schering Corp.) to 50 μg/ml and 20% (v/v) newborn-calf serum (Flow Laboratories or Dutchland Laboratories). Cells maintained in serum from some sources masked the expression of HA binding even though no serum was present during the binding assay. Screening of serum lots before use was therefore necessary. Alternatively, incubation of the cells for 60 min in a serum-free medium appeared to overcome this problem. RPMI/BSA is RPMI 1640 medium with 0.1% (w/v) BSA. PBS is 137 mM-NaCl/8 mM-sodium phosphate/2.7 mM-KCl/1.5 mM-potassium phosphate, pH 7.4. Tissue-culture dishes were from Corning or Falcon, and human plasma fibronectin was generously given by Dr. Gerald M. Fuller (University of Alabama, Birmingham, AL, U.S.A.). All other materials were of reagent grade.

Liver sinusoidal endothelial cells

Rat liver-cell suspensions were prepared by the collagenase-perfusion technique of Seglen [34] with some modifications [35,36]. Supernatants from the differential-centrifugation steps contain the non-parenchymal cells and were used as the starting material for the purification of LEC. Cells were pelleted by centrifugation at 450 *g* for 10 min at 4 °C, followed by resuspension in complete RPMI 1640 medium. This wash was repeated and LEC were isolated after centrifugation through discontinuous Percoll gradients as described by Eriksson *et al.* [17] and Smedsrod *et al.* [37,38]. Cells collected from the 25%/50% Percoll interface were seeded on to fibronectin-coated 35 mm-diameter culture dishes at 2×10^6 cells/plate in complete medium. Cell cultures were incubated at 37 °C in CO_2/air (1:19) atmosphere at 100% humidity. Experiments were conducted after the cells had been in culture for 8–24 h.

Measurement of surface and total LEC HA-receptor content

^{125}I-HA binding to LEC was measured at 4 °C. Cell cultures were washed three times with serum-free RPMI 1640 and chilled on ice. To conserve ^{125}I-HA and to keep non-specific binding low, a half-saturating concentration of ^{125}I-HA [6.8×10^{-8} M; $K_d \simeq 5.8$ (± 2.8) $\times 10^{-8}$ M; $n = 12$] [21] in 1 ml of RPMI/BSA was added, and each culture dish was incubated at 4 °C for 60 min. Cells were then washed rapidly three times (< 5 min total) with RPMI 1640 to remove unbound ^{125}I-HA, dissolved in 0.3 M-NaOH, and radioactivity and protein content were measured. Non-specific binding was assessed by using a 150-fold excess of non-radiolabelled HA in the binding mix. Specific binding is the total binding minus the non-

specific binding. To measure the surface and intracellular or only the surface HA-receptor activity, the binding assay was performed in the presence or absence respectively of 0.055% (w/v) digitonin added from a 1.4% (w/v) stock solution in absolute ethanol [32]. Specific binding of ^{125}I-HA was typically 70–85% for total cellular and 40–60% for cell-surface binding [21]. Since binding was done at the K_d, the binding obtained represents only half of the receptors present. The binding value was therefore doubled to estimate receptor number. Estimating receptor number on the LEC in this way produced results almost identical with the $B_{max.}$ obtained by Scatchard analysis [21].

Measurement of degraded ^{125}I-HA

Culture media (50 μl) containing ^{125}I-HA was diluted with distilled water and 250 μg of carrier HA to a final volume of 300 μl. An equal volume of 6% (w/v) CPC in water was added at room temperature and the solution was vigorously mixed to form a white precipitate. After 10 min the precipitate was collected by centrifugation in a Beckman Microfuge B for 3 min. Radioactivity in the supernatant and pellet was then measured. An increase in non-precipitable ^{125}I-HA with time indicates degradation of HA. Over 88% of the radioactivity in the ^{125}I-HA preparations was initially precipitable. Further details of this assay may be obtained from P.H.W. on request.

General

Protein was measured by the method of Bradford [39], with BSA as the standard, and DNA was quantified by the method of Labarca & Paigen [40], with calf thymus DNA as the standard. ^{125}I-HA radioactivity was measured in a Packard Multiprias 2 γ-radiation spectrometer and ^{3}H radioactivity was determined in a Beckman LS 7500 liquid-scintillation spectrometer using complete counting cocktail 3a70B from Research Products International Corp. For most experiments the points on the graph represent the averages of duplicate or triplicate results, and the S.E.M. was less than ± 5% in virtually all cases.

RESULTS

Kinetics of LEC ^{125}I-HA endocytosis

The accumulation of radiolabelled HA by LEC at 37 °C has been examined previously in a series of important studies by Eriksson *et al.* [17] and Smedsrod *et al.* [18]. These studies, however, measured total cell-associated radioactivity and did not distinguish endocytosed from surface-bound HA. Subsequently, Laurent *et al.* [20] used hyaluronidase to remove surface-bound HA from cells that had endocytosed HA for 10 min and found that 48% of the initially surface-bound HA was internalized. We were interested in the longer kinetics of HA internalization and processing using the unique ^{125}I-HA probe [31]. The accumulation of ^{125}I-HA by LEC at 37 °C in culture with time was therefore monitored. Both cell-associated and internal ^{125}I-HA were measured (Fig. 1). To accomplish this, a way to dissociate receptor-bound HA was needed. Unlike that of many of the other receptors characterized to date, the dissociation of HA from the endothelial receptor is not facilitated at low pH (e.g. pH 5.0). In fact, the amount of ^{125}I-HA bound and the apparent binding affinity actually increases, approx. 10–30-fold, at pH 5.0 [21,41]. However, a series of three 10-min washes with a high-salt pH 9.0 buffer usually removes about 70% of the surface-bound or intracellular ^{125}I-HA previously bound to intact or digitonin-permeabilized cells respectively (see, e.g., Fig. 1 and Table 1).

Internalization at 37 °C was linear with time and did not begin to reach a plateau within 90 min (Fig. 1), a

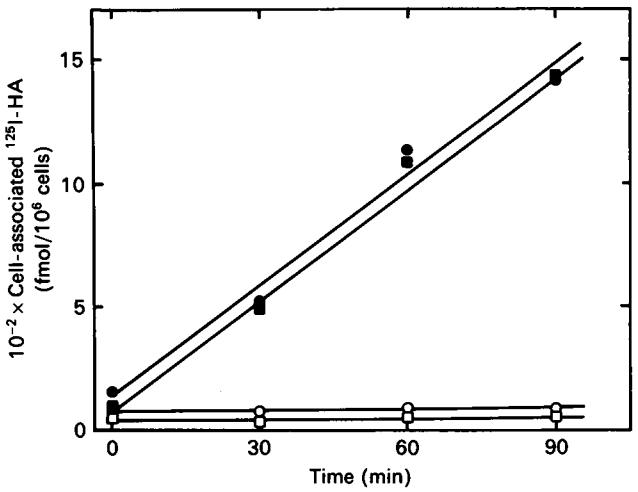

Fig. 1. Kinetics of ^{125}I-HA uptake by LEC in culture at 37 °C

LEC were incubated at 37 °C with 1.5 μg of ^{125}I-HA/ml with (○, □) or without (●, ■) 225 μg of non-radiolabelled HA. At the indicated times, cells were chilled to 4 °C, washed, and cell-associated ^{125}I-HA was measured (○, ●). Some cells were also washed three times for 10 min/wash at 4 °C with 0.5 M-NaCl/0.05 M-borate, pH 9.0, to dissociate surface-bound HA, and cell-associated ^{125}I-HA was then measured (□, ■).

Table 1. Quantification of endocytosed and surface-bound ^{125}I-HA

LEC in culture were incubated at 37 °C with 1.5 μg of ^{125}I-HA/ml with or without 225 μg of non-radiolabelled HA/ml for 90 min. Cells were then put on ice and non-cell-associated HA was removed by washing with 1 ml of PBS three times. The cells then had surface receptor–HA complexes dissociated by three 10 min washes at 4 °C with 0.5 M-NaCl/0.05 M-borate, pH 9.0. Cells were then permeabilized with 0.055% digitonin in PBS for 10 min on ice. Specifically cell-associated radioactivity released by the high-pH wash (surface-bound), and then subsequently released by the digitonin permeabilization (intracellular and free) or remaining with the permeable cells (intracellular and receptor-bound) was measured.

HA	^{125}I-HA (fmol/10^6 cells)	Percentage of total
Total cell-associated	1310	100
Surface-bound	22	2
Intracellular	1288	98
Intracellular free, released by digitonin	1134	87
Intracellular receptor-bound, after digitonin treatment	154	12

finding similar to that of Eriksson *et al.* [17], who used metabolically labelled HA. This indicates that the ^{125}I-HA, which has been modified only at the reducing end, behaves similarly to the unmodified metabolically labelled HA probes used by others. Under these conditions the rate of ^{125}I-HA binding to cell-surface receptors is very rapid [41] and essentially complete within 5 min. Surface-bound ^{125}I-HA was removed in this experiment from one set of cell samples using the high-salt pH 9.0 wash procedure. These dissociation conditions released only 2% of the ^{125}I-HA accumulated by LEC after 90 min. The remaining surface-bound ^{125}I-HA was only 0.7% of the total cell-associated ^{125}I-HA and was neglected. The resistance of accumulated ^{125}I-HA to these stripping conditions suggests that the cell-associated ^{125}I-HA is mostly intracellular. This conclusion was supported further by the release of 87% of the ^{125}I-HA accumulated in 90 min by treatment of the cells with 0.055% digitonin at 4 °C (Table 1). This digitonin treatment permeabilizes cells and releases soluble components, but releases only about 7% of the receptor–^{125}I-HA complexes preformed at 4 °C [41]. Therefore the digitonin treatment does not solubilize HA–receptor complexes from LEC, but permeabilizes the cells and allows internalized and dissociated ^{125}I-HA to be released from the cells. Receptor-bound HA remains with the permeable cells (Table 1). Internalization of ^{125}I-HA was inhibited by > 92% in the presence of a 150-fold excess of non-radiolabelled HA (Fig. 1). The steady-state rate of internalization in the experiment in Fig. 1 was calculated to be 146 molecules/s per cell at 3.4×10^{-8} M-^{125}I-HA. During the course of the experiment the amount of surface-bound ^{125}I-HA remained essentially constant, indicating a constant steady-state HA-receptor content on the cell surface during continuous endocytosis. In the subsequent experiments, the surface-stripping procedure was not routinely employed, since it was time-consuming and the percentage of surface-bound ^{125}I-HA is very small after 1–2 h of accumulation.

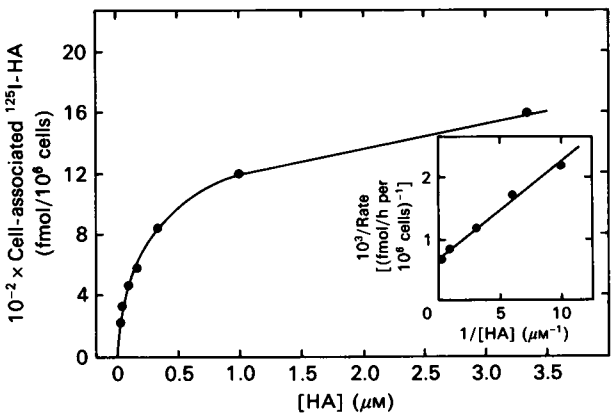

Fig. 2. Saturation of the rate of ^{125}I-HA internalization by LEC

LEC were incubated with 1.5 μg/ml of ^{125}I-HA at 37 °C for 1 h with increasing concentrations of non-radiolabelled HA. The cells were then washed and specific cell-associated radioactivity was measured by subtracting non-specific accumulation of radiolabel. Each point is the average for triplicate determinations. The inset is a double-reciprocal plot of the data. These data were obtained using the $M_r \sim 30000$ ^{125}I-HA probe.

Saturation of LEC endocytic rate at high concentrations of ^{125}I-HA

The rate of ^{125}I-HA internalization by LEC at 37 °C increased almost linearly with increasing concentrations of ligand until approx. 4×10^{-7} M (Fig. 2). A plateau was approached at HA concentrations greater than 3×10^{-6} M. The apparent K_m for the rate of internalization is about 0.4×10^{-6} M-HA. A double-reciprocal plot (Fig. 2, inset) was used to estimate the maximal rate of endocytosis. At saturation the maximal endocytic rate was 250 molecules/s per cell. Such a saturation effect is typical of a receptor-mediated endocytic process and would not occur if uptake was by a fluid-phase process. A fluid-phase component in the internalization of HA by LEC reported by others [18,42] could be due to the much larger HA probe and the necessarily lower molar concentration of HA used in those studies.

Specificity of ^{125}I-HA endocytosis by LEC

Various saccharides were assessed for their ability to prevent endocytosis of ^{125}I-HA by LEC at 37 °C (Fig. 3). An almost identical pattern of competition with these saccharides was observed for binding at 4 °C [21]. Of the various compounds examined, only three, namely HA, chondroitin sulphate and heparin, were efficient competitors of the endocytosis of ^{125}I-HA in LEC. Desulphated chondroitin sulphate and dextran sulphate also showed a significant ability to compete for endocytosis in these cells. Unrelated polyanions, however, such as DNA, RNA and polygalacturonic acid, were not effective competitors for endocytosis of ^{125}I-HA. Dextran, glucuronic acid, *N*-acetylglucosamine, insulin, *NN'*-diacetylchitobiose and haptoglobin were likewise unable to compete.

Endocytosis and degradation of ^{125}I-HA by LEC

The degradation assay we have employed requires that intact HA is efficiently precipitated while small degradation products remain in the supernatant. However, precise information on the extent of degradation required before HA is no longer precipitable with CPC is not available. Results from other studies indicate that HA oligosaccharides must be less than about 54 monosaccharides in length in order to be completely non-precipitable under the assay conditions used here (C. T. McGary & P. H. Weigel, unpublished work).

In the absence of cycloheximide, LEC in culture endocytose and accumulate HA in a linear manner for the first 3 h at 37 °C (Fig. 4). By 4 h, LEC reached a steady state between the accumulation of ^{125}I-HA and the release of ^{125}I-HA degradation products. A plateau in the level of cell-associated ^{125}I-HA was seen from 4 to 8 h. However, the total amount of ^{125}I-HA processed by these cells increased linearly for the duration of the experiment. By 6 h the LEC had processed a total of 4775 fmol of ^{125}I-HA/10^6 cells. This represented an average of 2875000 molecules/cell (Table 2). HA-receptor content was assessed in the same experiment in cells not exposed to ^{125}I-HA at 37 °C. Equilibrium binding of ^{125}I-HA was performed at 4 °C in the presence or absence of the permeabilizing detergent digitonin as described in the Experimental section. Only 40 fmol of ^{125}I-HA/10^6 cells were bound to the cell surface, whereas

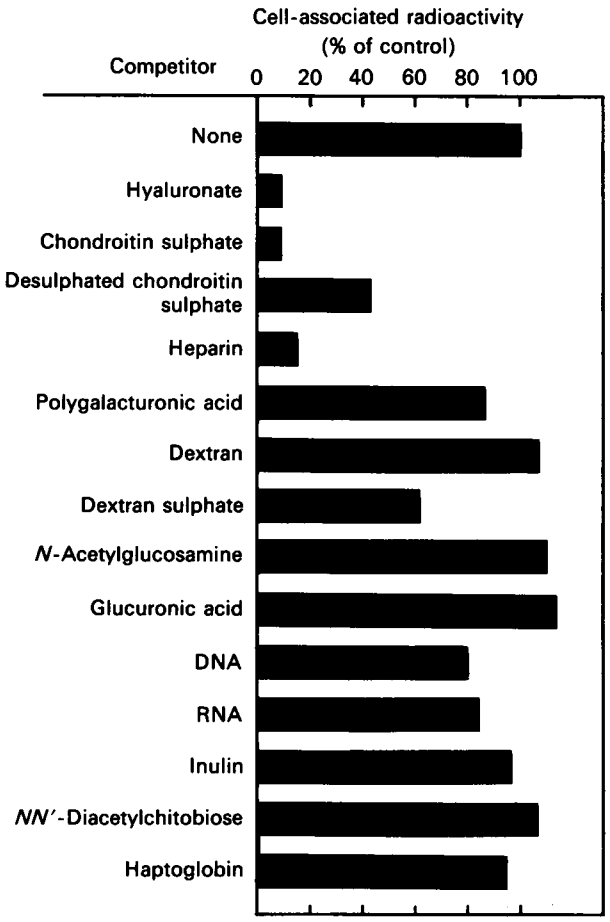

Fig. 3. Specificity of ^{125}I-HA internalization by cultured LEC

LEC were incubated with 2×10^{-7} M (6 μg/ml)-^{125}I-HA with or without 600 μg of the indicated competitors/ml for 1 h at 37 °C. The cells were washed, solubilized, and radioactivity was determined. The amount of cell-associated radioactivity is expressed as a percentage of the control without competitor. Each bar represents the average of triplicate determinations. At 600 μg/ml the monosaccharide concentration is only 3 mM, which is an insignificant increase in the osmolarity of the media. These data were obtained using the $M_r \sim 30000$ ^{125}I-HA probe.

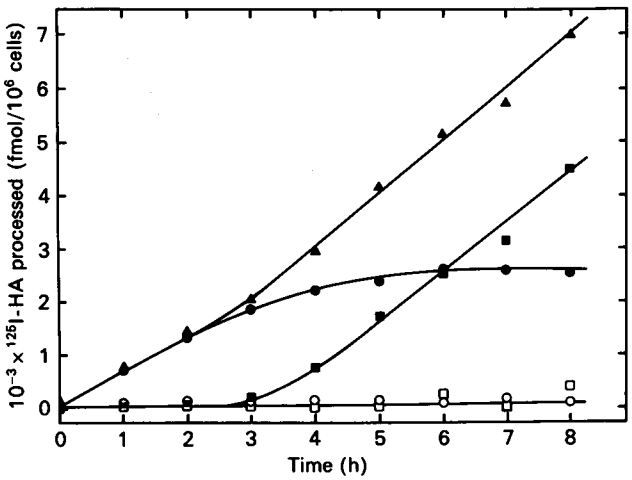

Fig. 4. Long-term kinetics of internalization and degradation of ^{125}I-HA by LEC

Cultured cells were washed three times with serum-free medium and then incubated in 1 ml of RPMI/BSA with 3 μg of ^{125}I-HA/ml plus (○, □) or minus (■, ●, ▲) 450 μg of non-radiolabelled HA/ml at 37 °C. At the indicated times, culture media was removed and assayed for degraded ^{125}I-HA (□, ■). The cells were then washed three times with media at 4 °C, dissolved in NaOH, and cell-associated radioactivity (○, ●) and protein were measured. Processed ^{125}I-HA (▲) was calculated as the sum of degraded and cell-associated HA. The zero-time point represents specific surface binding of ^{125}I-HA at 4 °C determined as described in the Experimental section. Total cellular specific binding capacity for ^{125}I-HA was also determined at 4 °C, using digitonin as described in the Experimental section. Each point is the average of duplicates.

180 fmol of ^{125}I-HA/10^6 cells were bound to both the surface and internal HA receptors. Assuming that one HA molecule is bound by one receptor and that one-half of the receptors are occupied, there are about 22×10^4 total HA receptors/cell; about 4.9×10^4 receptors/cell are on the cell surface.

As Table 2 shows, 13 times more ^{125}I-HA was processed than the total cell complement of HA receptors, which indicates that the HA receptors may be recycled. However, a very rapid replacement of receptors that were degraded after they functioned could also give the same result. This experiment was therefore repeated in the presence of cycloheximide to inhibit protein synthesis *de novo* and rule out this possibility (Fig. 5). The kinetics of cell accumulation of ^{125}I-HA and the release of degraded products were virtually the same as those seen in Fig. 4. However, the steady-state rate of ^{125}I-HA uptake and degradation and the final steady-state amount of intracellular ^{125}I-HA accumulated by the cells were about half of that when no cycloheximide was present (Fig. 4), even though the cellular HA-receptor content was unchanged (Table 2). In addition, a cessation in the processing of ^{125}I-HA was seen in the presence of cycloheximide after 5 h (Fig. 5) that was not seen in the absence of the drug. This plateau corresponded to the time when cell viability also began to decrease, presumably owing to the prolonged inhibition of protein synthesis. Nonetheless, as in Fig. 4, in the presence of cycloheximide the LEC were still able to process 6-fold more ^{125}I-HA than their total cellular HA-receptor number. In a separate experiment the rate of protein synthesis in LEC with and without 10^{-4} M-cycloheximide was assessed by the ability of the cells to incorporate NET-250 ^{3}H-labelled L-amino acids into trichloroacetic acid-precipitable material. This drug concentration inhibited the rate of ^{3}H incorporation by 92.6%.

It is possible that LEC, like hepatocytes [43], exhibit a deiodinase activity and that the measured degradation of ^{125}I-HA is actually the release of free iodide by these cells. Gel-filtration chromatography with Bio-Gel P-2 was used to separate intact ^{125}I-HA and degradation products from any free iodide that may have been present. Even after the cells had processed ^{125}I-HA for 24 h, no radioactivity was eluted at the position of KI (results not shown). Therefore the CPC degradation assay results

Table 2. Ratios of processed ^{125}I-HA to HA-receptor activity in LEC

Data are summarized from Figs. 4 and 5 at the 6 h time point. The surface and total HA-receptor contents of the cells in each experiment were measured as described in the Experimental section. For comparison with the Figs., 100 fmol/10^6 cells is approx. equivalent to 60000 sites/cell.

	^{125}I-HA processed at 37 °C in 6 h (molecules/cell)	
	−Cycloheximide	+Cycloheximide
	2875000	1605000
	^{125}I-HA bound at 4 °C (molecules/cell)	
Receptor(s)	−Cycloheximide	+Cycloheximide
Surface	48600	41200
Surface and internal	219000	267600
	Ratio of processed ^{125}I-HA/HA receptors	
Receptor(s)	−Cycloheximide	+Cycloheximide
Surface	59	39
Surface and internal	13	6

Table 3. Effect of exogenous HA on the HA-receptor activity of LEC in the presence of cycloheximide

LEC cultures were incubated in RPMI 1640/BSA with or without 3 μg of non-radiolabelled HA/ml in the presence of 10^{-4} M-cycloheximide. At the indicated times, the cells were chilled to 4 °C, permeabilized with 0.055% digitonin and the bound non-radiolabelled HA was removed by treatment with 0.5 M-NaCl/0.05 M-sodium borate, pH 9.0, as described in Table 1. Functional-receptor content was then measured by the ability of the permeable cells to specifically bind ^{125}I-HA at 4 °C as described in the Experimental section. Values are the averages of duplicates.

Time (h)	Total cellular specific ^{125}I-HA-binding capacity (% remaining)	
	−HA	+HA
0	100	100
2	105	105
4	97.3	104
6	99.8	109

measured HA breakdown by LEC and not just the release of free iodide.

LEC HA-receptor content during ^{125}I-HA processing

An additional test for receptor recycling is to determine whether the metabolic half-life of the receptor is unaffected by the presence of ligand. The receptors are therefore not turned over or degraded more quickly when they function. Such an experiment usually requires immunoprecipitation using a specific antibody, which is presently unavailable for this receptor. Therefore, an alternative experiment was performed. The HA-receptor content of LEC processing HA for 6 h in the presence of 10^{-4} M-cycloheximide was compared with the receptor content in cells that were not processing HA. To accomplish this, the cell samples were treated as described in Fig. 1 to remove receptor-bound non-radiolabelled HA from permeabilized cells. The ability of these permeable stripped cells to bind ^{125}I-HA was then determined at 4 °C. If LEC degrade HA receptors after they endocytose HA, then one would expect that cells processing HA in the presence of cycloheximide would lose HA-receptor activity with time, whereas cells not processing HA would maintain their HA-receptor content. There was no significant difference in the HA-receptor content of LEC with time whether they were processing or not processing HA (Table 3). This result suggests that the metabolic turnover of HA receptors does not increase when they function.

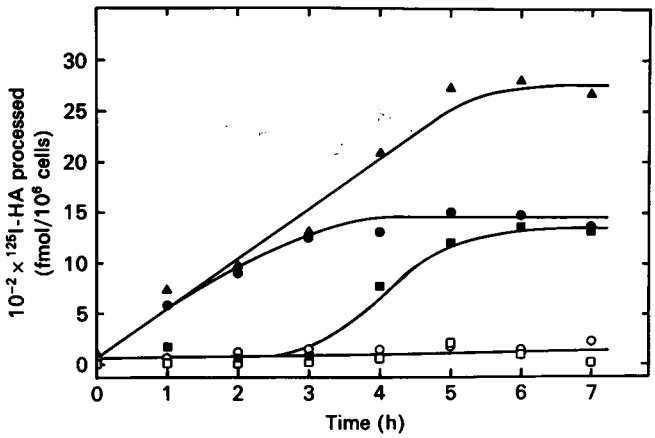

Fig. 5. Long-term kinetics of internalization and degradation of ^{125}I-HA by LEC in the presence of cycloheximide

LEC were incubated in RPMI 1640/BSA plus 10^{-4} M-cycloheximide for 30 min at 37 °C before the start of the experiment, and this drug concentration was maintained for the duration of the experiment. Cultured cells were washed three times with serum-free medium and then incubated in 1 ml of RPMI/BSA with 3 μg of ^{125}I-HA/ml plus (○, □) or minus (■, ●, ▲) 450 μg of non-radiolabelled HA/ml at 37 °C. At the indicated times, culture media was removed and assayed for degraded ^{125}I-HA (□, ■). The cells were then washed three times with media at 4 °C, dissolved in NaOH, and cell-associated radioactivity (○, ●) and protein were measured. Processed HA (▲) was calculated as the sum of degraded and cell-associated HA. The zero-time point represents specific surface binding of ^{125}I-HA at 4 °C determined as described in the Experimental section. Total cellular specific binding capacity for ^{125}I-HA was also determined at 4 °C using digitonin as described in the Experimental section. Each point is the average of duplicates.

Function of intracellular LEC HA receptors

Weigel & Oka [44] concluded that the intracellular asialoglycoprotein receptors exposed by digitonin in hepatocytes can function during continuous endocytosis.

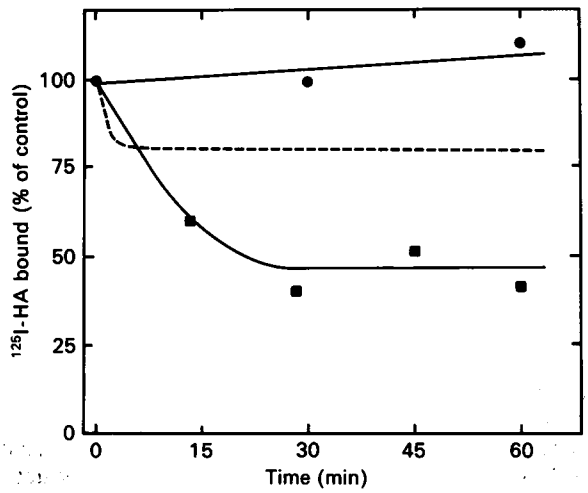

Fig. 6. Occupancy of intracellular HA receptors in LEC processing extracellular HA at 37 °C

LEC were incubated at 37 °C with (■) or without (●) 100 μg of HA/ml. At the indicated times the cells were put on ice and total cellular receptor content was measured using 1.5 μg of ^{125}I-HA/ml and 0.055% digitonin. A 10 min binding assay was used, since the dissociation of receptor–HA complexes is rapid [21,41]. Cells were washed three times, and bound radioactivity was measured as described in the Experimental section. Each point is the average of duplicates. The broken line represents 100% occupancy of the surface receptors.

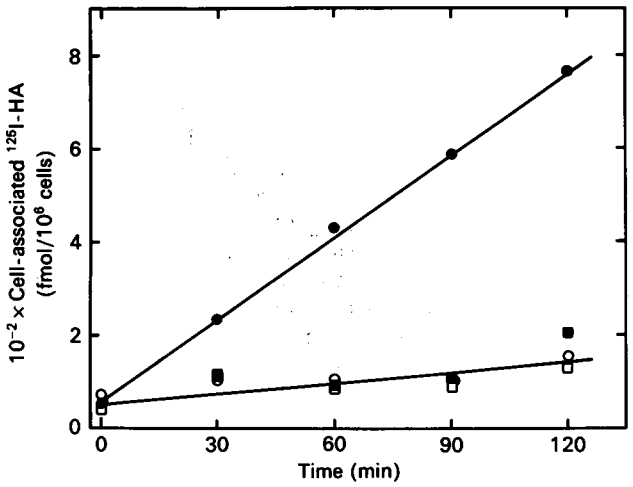

Fig. 7. Effect of hyperosmolarity on LEC ^{125}I-HA endocytosis

LEC were incubated at 37 °C with 1.5 μg of ^{125}I-HA/ml with (○, □) or without (●, ■) 225 μg non-radiolabelled HA and in the presence (□, ■) or absence (○, ●) of 0.4 M-sucrose. At the indicated times cells were washed, and cell-associated ^{125}I-HA was measured as described in the Experimental section.

This was done by demonstrating that the receptors become occupied when the cells process extracellular non-radiolabelled ligand at 37 °C. Receptor occupancy was assessed by measuring the loss of the total cellular ability to bind the radiolabelled ligand at 4 °C in the presence of digitonin. We carried out the same experiment with LEC and HA (Fig. 6). After 30 min of HA uptake at 37 °C, about 65% of the total cellular HA-receptor population was occupied. Since 75% of the total cellular HA receptors are internal, then at least 50% of the intracellular receptors became occupied with unlabelled ligand. This result indicates that the intracellular receptors function in some capacity during the endocytic process. The interpretation that the cells processing HA lose receptor activity because the receptors have been degraded is not tenable, since the wash conditions that dissociate HA from its receptor restore the lost binding activity (Table 3).

Effect of hyperosmolarity on ^{125}I-HA endocytosis by LEC

Receptor-mediated endocytosis in other recycling receptor systems occurs by a coated-pit pathway [23]. Sucrose-induced hyperosmolarity was recently shown to stop receptor-mediated asialoglycoprotein endocytosis, but not fluid-phase endocytosis, in rat hepatocytes [45,46]. Clathrin-coated pits invaginate to form internalized coated vesicles. The clathrin is then dissociated [47] and used to re-form new coated pits. Thus a clathrin cycle exists between assembled coated pits and free clathrin [47,48]. Hyperosmolarity has also been shown to interfere with the recycling of clathrin-coated pits in fibroblasts [49]. We therefore investigated the effect of 0.4 M-sucrose on ^{125}I-HA endocytosis by LEC (Fig. 7). Control cells internalized ^{125}I-HA in a linear fashion for 2 h. However, in the presence of 0.4 M-sucrose, the rate of ^{125}I-HA endocytosis was inhibited by > 90%, to the same level as non-specific internalization. Sucrose did not inhibit the binding of ^{125}I-HA to LEC, and cell viability was maintained throughout the experiment (results not shown). This result is consistent with a coated-pit pathway for endocytosis mediated by this HA receptor.

DISCUSSION

Accumulation of ^{125}I-HA at 37 °C by LEC follows kinetics typical of an endocytic internalization of receptor-bound ligand. The inability to release cell-associated HA at 4 °C with a treatment that dissociates 70% of preformed ^{125}I-HA–receptor complexes suggests that the vast majority of the accumulated HA is intracellular (Fig. 1). This is further substantiated by the ability of digitonin to permeabilize LEC and to release 87% of the ^{125}I-HA accumulated after 90 min. The release of cell-associated ^{125}I-HA after the digitonin permeabilization also indicates that dissociation of ^{125}I-HA–receptor complexes occurs after endocytosis. Such a dissociation event is necessary for a system that degrades ligand and recycles the receptor. Dissociation in this HA-receptor system is probably not mediated by low pH, since this does not facilitate disruption of preformed ^{125}I-HA–receptor complexes. The HA receptor also has no bivalent-cation requirement. The mechanism of intracellular receptor–ligand dissociation is therefore unknown and may be novel. An alternative possible mechanism to explain ligand dissociation is that the HA receptor may be inactivated after internalization by a covalent modification or by interaction with another regulatory molecule. The inactive receptor would then release the bound HA and each molecule could follow its distinct intracellular itinerary. We have recently described such an inactivation/re-activation cycle in the hepatic asialoglycoprotein-receptor system [50,51].

The clearance of HA from plasma due to its endo-

cytosis by LEC has been described by others as a combination of both fluid-phase and receptor-mediated processes [18,42]. This conclusion was based on the observation that saturation of endocytosis by LEC at 37 °C did not occur at the same HA concentration required to saturate binding at 7 °C [18,20]. We have also observed that near the HA concentration ($\sim 10^{-7}$ M) needed to saturate binding at 4 °C, the endocytosis of HA at 37 °C does not saturate. However, an approach to the saturation of endocytosis at 37 °C (Fig. 2) was observed at higher ^{125}I-HA concentrations (10^{-6} M). The rate of specific ^{125}I-HA endocytosis by LEC appears to saturate at high HA concentrations and is virtually completely inhibited by an excess of non-radiolabelled HA; these are both characteristics of a receptor-mediated process. Furthermore, the maximum endocytic rate of 250 molecules of HA/s per LEC compares well with the maximum values of about 700 and 550 molecules/s per cell for the hepatocyte asialoglycoprotein receptor [52] and the macrophage mannose receptor [53] respectively. When normalized for the receptor content per cell, these three different receptor-recycling systems are all essentially identical. We conclude that there is not a significant fluid-phase component ($\leqq 5\%$) to the LEC internalization capacity of the ^{125}I-HA, particularly at the low concentrations of ^{125}I-HA used in the present study. The size of this ^{125}I-HA probe ($M_r \sim 44000$) is also much smaller than that used in the above studies ($M_r \sim 400000$) and could account for the slightly different results. The concentration difference required to saturate binding as opposed to endocytosis may in part be related to the different temperatures at which the two processes are measured. At 37 °C, the binding equilibrium between HA and receptor may be different than at 4 °C. Also, owing to rapid internalization of HA–receptor complexes at 37 °C, equilibrium between association and dissociation of HA at the cell surface is probably not attained. Additionally, in other receptor systems endocytosis has been shown to saturate at higher ligand concentrations than those required to saturate binding [52].

The ability of LEC to endocytose ^{125}I-HA is not absolutely specific for HA, since chondroitin sulphate and heparin also compete very well with ^{125}I-HA for both binding [21,41] and endocytosis (Fig. 3). Other investigators have also demonstrated that competition for HA endocytosis by LEC occurs with chondroitin sulphate but not with heparin [18]. However, the size of the [^{3}H]HA used in those experiments was large ($M_r \sim 400000$) compared with the ^{125}I-HA used in our experiments ($M_r \sim 44000$). This suggests that heparin might compete effectively for endocytosis of small HA molecules but not for very large HA molecules. We also conclude, as Laurent *et al.* [42] have proposed, that the LEC probably also function *in vivo* to remove these other glycosaminoglycans from the circulation by using the same receptor.

The processing of ^{125}I-HA by LEC (i.e. the internalization, degradation and release of degraded products) was linear for at least 8 h (Fig. 4), although degraded ^{125}I-HA products were not detectable in the medium before 3 h. In other experiments (C. T. McGary & P. H. Weigel, unpublished work) we have found that this delay does not represent a lag in the degradation process, however, because 50% of the intracellular ^{125}I-HA is degraded and this pool increases for 2–3 h before products appear in the medium. This result indicates that a surface-bound hyaluronidase activity cannot be responsible for the observed degradation. In other receptor systems that internalize and degrade their respective ligands, such as the asialoglycoprotein-receptor system of hepatic parenchymal cells, the release of degradation products from the cells begins almost immediately [36]. Using metabolically labelled HA, others have detected the release of HA degradation products (acetate and lactate) by LEC into the culture media as soon as 30 min [18]. The reason for the longer delay in the appearance of degradation products in the present study is not clear. The ^{125}I-HA probe employed here has been modified and radiolabelled only at the reducing end of the molecule. Since only the radiolabel is being monitored, the observed lag time could reflect the accumulation within the cell of the chemically modified reducing end of the molecule after HA degradation. The radioactive end product of degradation, which is not a normal cell molecule, may not be as readily released from lysosomes. The structure of the modified HA reducing end, in fact, is structurally similar to groups that others have coupled to macromolecules in order to concentrate the degradation products in lysosomes and thereby localize the tissue and cellular site of degradation [54].

When the cell-surface and internal HA-receptor number is compared with the amount of ^{125}I-HA processed per cell in 6 h, it is clear that receptor re-utilization must occur. Even if all the intracellular HA receptors were involved in the endocytic cycle (i.e. all cellular HA receptors are functional and functionally equivalent), then each HA receptor would have still been used about 13 times to account for the total amount of ^{125}I-HA processed. A similar situation occurred also in the absence of protein synthesis. Each receptor was used 6 times, indicating that recycling of receptors must occur. If HA receptors were degraded after internalizing HA and were replaced by newly synthesized receptors, we estimate that at a saturating HA concentration the half-life of functioning receptors in the absence of protein synthesis would only be about 6.1–27.7 min (depending on whether respectively none or all of the intracellular receptors were functional; Table 2). In fact no degradation of HA receptors was observed within 6 h in the presence of cycloheximide (Table 3).

At least 50% of the intracellular HA receptors (65% of the total surface and intracellular receptors) in LEC become occupied with HA of extracellular origin during continuous endocytosis at 37 °C (Fig. 6). However, this is an underestimate, owing to the rapid rate of dissociation of HA ($M_r \sim 44000$) from the receptor [21,41]. During the 10 min binding of ^{125}I-HA at 4 °C used in this experiment, about 20% of the preformed HA–receptor complexes would be expected to dissociate owing to this rapid off rate. Therefore we estimate that at least 70% of the intracellular HA receptors in LEC function during endocytosis.

Assuming that all cellular LEC HA receptors participate in the receptor cycle during endocytosis, then, on the basis of the data in Table 2, we calculate that, within 28 min, all of the HA receptors would have been through the cycle an average of at least once. If HA receptors were degraded after endocytosis, then when protein synthesis is inhibited and the cells are allowed to process HA, all HA receptors would be degraded within 30 min. However, several different experiments in the presence of cycloheximide show there is no loss of HA receptor

activity even after 6 h, a time when at least six rounds of endocytosis had occurred (Table 3). There is, therefore, no indication that HA receptors are degraded during continuous endocytosis, an observation further supporting a recycling pathway.

Other recycling endocytic systems, such as the asialoglycoprotein and low-density-lipoprotein receptors, utilize coated-pit pathways [23]. Hyperosmolarity induced with sucrose interrupts the coated-pit pathway in hepatocytes [45,46] and fibroblasts [49]. For hepatocytes in suspension, 0.2 M-sucrose was optimal for disruption of coated-pit endocytosis, but 0.4 M-sucrose was needed to produce a maximal effect in cultured hepatocytes or fibroblasts [46,49]. Thus we used 0.4 M-sucrose for cultured LEC. If the coated pits of LEC are similarly disrupted by sucrose-induced hyperosmolarity, an inhibition of endocytosis should be observed under such conditions. The ability of 0.4 M-sucrose to inhibit ^{125}I-HA endocytosis to non-specific levels (Fig. 6) strongly suggests that a coated-pit pathway is involved. Another interpretation would be that endocytosis could still occur, but that 0.4 M-sucrose prevents ^{125}I-HA from binding to its receptor. However, binding of ^{125}I-HA to LEC at 4 °C was not affected by 0.4 M-sucrose; thus receptor–HA complexes are formed. The effect of sucrose, therefore, is on endocytosis and not on binding.

We conclude that endocytosis of HA by LEC occurs through a receptor-mediated process and that a receptor-recycling pathway exists. Furthermore, it appears that a coated-pit pathway is involved and that HA, heparin and chondroitin sulphate are all endocytosed by the same receptor in LEC. At least 70% of the intracellular receptor population is involved in this recycling pathway.

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