Synthesis and release of hyaluronic acid by Swiss 3T3 fibroblasts

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Hyaluronic acid (HA) and its synthesis were studied in intact Swiss 3T3 mouse fibroblasts and isolated membranes. HA chains in culture medium, attached to cells and in isolated membranes, were determined to possess average M_r values of 5.2×10^6 , 1.8×10^6 and 0.14×10^6 respectively. Log cells were determined to possess 680 000 HA molecules/cell, and to release 120 000 HA chains/h. The time required for intact cells to synthesize and release a complete HA chain was approximately 4 h, with elongation proceeding at a rate of 57 dimers/min. The amount of cell-associated HA of various cell populations correlated strongly

with their rate of HA release into culture media and with the HA synthetase activity determined for their membranes. Prevention of protein synthesis with cycloheximide decreased the rate of HA synthesis of log cells and HA synthetase activity of isolated membranes by 50 % within 2–3 h. Because of the similarity between the biological lifetime of HA synthetase and the time required to synthesize a HA chain, we propose a model where each synthetase makes only one HA chain; after synthesis of a complete HA chain, HA synthetase activity is terminated as its HA chain is released from the cell.

INTRODUCTION

Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan (GAG), composed of alternating glucuronic acid and *N*-acetylglucosamine residues. HA has characteristics common to other GAGs, including the presence of an amino sugar in a repeating dimer, the absence of any branching, a high negative charge and a role as a major constituent of the extracellular matrix. HA does, however, possess several characteristics which distinguish it from other members of this classification. It is not sulphated and, with an M_r reaching 10^6 – 10^7 , the size of HA is one to two orders of magnitude larger than any of the other GAGs [1]. Additionally, normal GAG synthesis involves elongation at the Golgi by sequential addition of sugars to a core protein, followed by export of the finished product to the cell surface. By contrast, HA synthesis occurs at the plasma membrane and the nascent chain is extruded directly into the extracellular matrix [2,3]. No core protein has yet been shown to be involved in HA synthesis.

In addition to curiosity generated from its unique standing in the GAG family, interest in HA has been further stimulated by observations that a variety of processes requiring cell motility are preceded by a rise in locally produced HA [4]. It is believed that as this HA becomes increasingly hydrated, it expands the extracellular space [5]. This process mechanically weakens intercellular binding and increases the distance separating cells, a situation facilitating cell detachment. In addition to promoting detachment, the HA can also provide hydrated tracks along which the migration of detached cells can be directed to specific destinations, as observed in wound repair and embryonic morphogenesis [5,6]. The involvement of HA in cell detachment and migration is not limited to normal cellular processes. HA is believed to play a key role in pathological events such as cancer, where it may contribute to tumour invasion and metastasis. Indeed, many carcinomas have been observed to be surrounded by an extracellular matrix rich in HA [7], and the expression of

an HA receptor (CD44) on the cell surface of tumours is a good indicator of metastatic potential [8]. Interestingly, HA synthesis is not performed by the carcinomas directly, but by neighbouring fibroblasts in response to factors produced by the carcinomas [7,9].

The focus of the research presented herein is to study the synthesis and release of HA, with the aim of gaining a better understanding of the cellular regulation of HA synthesis and, hence, the processes involved in cell detachment and migration. A fibroblast cell line was chosen for study because, in the interaction between epithelial and mesenchymal cells, it is the fibroblasts that are directed to synthesize HA. In this report we determined the average size of the HA chains that are found associated with the cell surface, are released into the culture media and are present in isolated membrane preparations of Swiss 3T3 fibroblasts. From these data we were able to calculate the number of HA chains per cell, their rates of synthesis and release, the probable volume occupied by the halo of HA surrounding a cell and an estimate of the HA synthetase activity retained by isolated membranes. Moreover, the similarity between the biological lifetime of HA synthetase and the time necessary for the synthesis of a complete HA chain, as well as other data presented herein, suggest that each molecule of HA synthetase is responsible for the synthesis of only one HA chain.

EXPERIMENTAL

Materials

Riboflavin, UDP-GlcNAc, and UDP-GlcA were obtained from Sigma Chemicals, St. Louis MO, U.S.A. Hyaluronidase (*Streptomyces hyalurolyticus*) was purchased from Sigma Chemicals and from Calbiochem, La Jolla, CA, U.S.A. NEN Products, Boston MA, U.S.A. was the source for [^{14}C]GlcNAc (specific activity 60 mCi/mmol), [^{14}C]UDP-GlcA (specific activity 285.2 mCi/mmol), L-[^{14}C (U)]amino acid mixture (specific activity 50 mCi/matom carbon) and Aquassure liquid scintillation cock-

Abbreviations used: HA, hyaluronic acid; GAG, glycosaminoglycan; CS, calf serum; LMW, low-molecular weight; HMW, high-molecular weight; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EM, electron micrograph, V_0 , column void volume; V_t , column total volume.

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tail. [Methyl-³H]thymidine (specific activity 54.4 mCi/mmol) was purchased from ICN Biochemicals, Costa Mesa, CA, U.S.A. The kits used for quantification of HA were purchased from Pharmacia Diagnostics, Uppsala, Sweden. The Sephadex and Sepharose chromatography gels, high-molecular weight (HMW) and low-molecular weight (LMW) protein standards and Blue Dextran were made by Pharmacia Fine Chemicals, Uppsala, Sweden. The tissue culture materials, Dulbecco's modified Eagle's medium (DMEM), 0.5 % trypsin/0.1 % EDTA, L-glutamine and normal saline were supplied by Quality Biological Inc., Gaithersburg, MD, U.S.A. while filtered calf serum (CS) and gentamicin sulphate were supplied by Colorado Serum Company, Denver, CO, U.S.A. and BioWhittaker, Walkersville, MD, U.S.A.

Tissue culture

Murine Swiss 3T3 fibroblasts were purchased from American Type Culture Collection (Rockville, MD, U.S.A.), removed from cryogenic storage and used for experimentation between passages 5 and 20. On day 1 of subculture, 2×10^5 cells were seeded per flask (Nunc T-75 cm²) in 15 ml of 10 % CS-DMEM, 2 mM glutamine and 50 µg/ml gentamicin. Cell number was determined using a Model Z_{BI} Coulter Counter (Coulter Electronics, Hialeah, FL, U.S.A.). Flasks were incubated at 37 °C in humidified air containing 12.5 % CO₂. For experiments requiring log-phase cells, T-75 cm² culture flasks containing approximately 1×10^6 cells were used 3 days after subculture. For experiments requiring quiescent cells, the medium was replaced on day 3 with fresh 10 % CS-DMEM and incubation continued for an additional 7 days, at which time the cells achieved confluency and became quiescent due to serum starvation. Quiescence after 10 days of subculture was determined by [³H]thymidine incorporation for the 24 h after the start of day 10. A 20-fold difference in [³H]thymidine incorporation between CS-stimulated and non-stimulated cells was used as the criterion for the quiescent state. These quiescent cells were used in experiments which required a stationary cell population, and typically contained (2–3) $\times 10^6$ cells per flask. Cells to be treated with cycloheximide were rinsed once with DMEM lacking CS, followed by the addition of 10 % CS-DMEM containing 10 µg/ml cycloheximide. Some experiments included the addition of [¹⁴C]GlcNAc at this point. Aliquots of medium from treated and non-treated cells were removed at various times, made 1 % in SDS, boiled for 3 min and stored at 4 °C. Cells to be analysed for HA content were treated with trypsin, made 1 % in SDS, boiled for 3 min and stored at 4 °C.

Membrane isolation and determination of HA synthetase activity

Cells were detached by treatment with 0.5 % trypsin/0.1 % EDTA and washed twice with ice-cold 10 mM Hepes-NaOH, pH 7.2, containing 0.25 M sucrose and 0.5 mM dithiothreitol (DTT). Cells were pelleted between washes by a 10 min centrifugation at 1000 g at 4 °C. The cell pellet was suspended in 0.25 ml of ice-cold 10 mM Hepes-NaOH, pH 7.2, containing 0.25 M sucrose and 0.5 mM DTT, and vortexed. This was followed by sonication (VibraCell, Sonics and Materials Inc., Danbury, CN, U.S.A.) on ice using a microtip at its output limit, for 7 $\times$ 1 s bursts, using a 40 % duty cycle. Breakage was confirmed using a phase-contrast microscope. Unbroken cells and large debris were removed by the addition of 0.75 ml of ice-cold 10 mM Hepes-NaOH, pH 7.2, containing 0.25 M sucrose and 0.5 mM DTT, followed by 2000 g centrifugation at 4 °C for 10 min. The supernatant was removed and centrifuged at

200 000 g for 45 min at 4 °C in a Beckman TLA 100.2 rotor. The supernatant was discarded and the pellet resuspended in 0.5 ml of ice-cold 25 mM Hepes/NaOH, pH 7.1, containing 0.25 M sucrose and 5 mM DTT. The resuspended pellet was centrifuged again for 45 min at 200 000 g at 4 °C. The supernatant was discarded and the pellet suspended in 0.5 ml of ice-cold 25 mM Hepes-NaOH, pH 7.1, containing 0.25 M sucrose and 5 mM DTT. An aliquot was removed and assayed for protein content by the method of Bradford [10], using reagents obtained from BioRad Laboratories (Hercules, CA, U.S.A.).

A known amount of protein, typically 100 µg, was then assayed for HA synthetase activity by incubation in 30 µl of a mixture containing 15 mM MgCl₂, 2 mM UDP-GlcNAc, and 0.1 mM of [¹⁴C]UDP-GlcA (1 µCi). The reaction mixture was incubated at 37 °C for 1 h, unless otherwise noted, and the reaction terminated by addition of SDS to a 1 % concentration, followed by boiling for 3 min. The samples were then stored at 4 °C for later analysis by Sephadex G-200 or Sepharose 4B chromatography. After chromatography, the void-volume (V_0) ¹⁴C contents of the samples were tabulated. The HA synthetase activity of each membrane sample (nmol HA dimer/h per mg protein) was then calculated based on its total V_0 ¹⁴C d.p.m., the amount of membrane protein assayed, the incubation time and the specific activity of the [¹⁴C]GlcA used.

Hyaluronic acid determination

Hyaluronic acid in samples was measured with Pharmacia's HA 50 analysis kit. The method is based on an ¹²⁵I-labelled HA binding protein assay developed by Tengblad and Laurent [11,12], which determines the amount, in ng, of HA present in a sample, but gives no information about the number of HA chains or their lengths. The test is capable of detecting HA chains possessing ≥ 8 dimers ($M_r \geq 3000$). All test manipulations were performed as prescribed by Pharmacia. A Packard Auto-Gamma 5000 series Gamma Counter was used to determine ¹²⁵I content.

Determination of media HA content

Media samples were allowed to warm to room temperature for 1 h, vortexed, and filtered through a Gelman 0.45 µm sterile Acrodisk filter (Gelman Sciences, Ann Arbor, MI, U.S.A.). A 2 ml aliquot from each sample was applied to a Sepharose 4B column (inner diameter 2.5 cm $\times$ 32 cm) and eluted with 50 mM Tris-HCl, pH 7.1, at a flow rate of 0.3 ml/min. Blue dextran and riboflavin were used to determine V_0 and total volume (V_t) of the column and Pharmacia's HMW and LMW protein standards were used to obtain a selectivity curve for proteins. Fractions of 2 ml were collected and assayed for ¹⁴C content, using a Packard 2200CA Tri-Carb Series Liquid Scintillation Analyzer, and/or HA content, with Pharmacia's HA 50 kit.

Determination of cellular HA content

Cells were washed once with normal saline, followed by treatment with 2 ml of 0.5 % trypsin/0.1 % EDTA. The suspended cells were disrupted by addition of 0.2 ml of 10 % SDS, followed by boiling for 3 min. Samples that could not be processed immediately were stored at 4 °C. After storage, the samples were allowed to warm to room temperature for 1 h, vortexed, and filtered through a Gelman 0.45 µm sterile Acrodisk filter. The samples were applied to a Sephadex G-25 column (inner diameter 1.5 cm $\times$ 27 cm) and eluted with 50 mM Tris/HCl, pH 7.1, at a flow rate of 3 ml/min. This was done to remove SDS, which interferes with determination of HA by the Pharmacia method. Fractions of 1 ml were collected and assayed for HA content.

Determination of membrane HA content

Cells were washed once with normal saline, followed by treatment with 2 ml of 0.5 % trypsin/0.1 % EDTA, as in the case of cellular HA samples. At this point, however, the cells are disrupted by sonication and the membranes partially purified by ultracentrifugation, as described earlier in the section Membrane isolation and determination of HA synthetase activity. After determination of protein content, an aliquot of protein (1–10 mg) was made 1 % in SDS, boiled for 3 min and applied to a Sephadex G-200 column after cooling. The resulting fractions were assayed for HA content, with Pharmacia's HA 50 kit.

Electron microscopy

Electron micrographs (EMs) of HA samples were determined by Program Resources Incorporated/DynCorp, Frederick, MD, U.S.A. using a modified Kleinschmidt [13] technique. The hypophase consisted of 12 % formamide and 0.04 % n-octyl- β -D-glucopyranoside in 10 mM Tris/1 mM EDTA, pH 8.5. The hyperphase consisted of the appropriate amount of sample diluted with doubly distilled water and 20 μ g/ml of cytochrome *c*, which was added before spreading. A 125 mM ammonium acetate solution was applied to 1 in $\times$ 3 in glass slides with a squirt bottle and allowed to air dry. A glass slide was placed in a Teflon-coated boat filled with hypophase and between Teflon-coated bars at a 45° angle. The hyperphase was slowly released onto the glass slide by moving a syringe from left to right. Four areas of sample were lifted from the hypophase surface with 300 mesh grids coated with 3.5 % Parlodian support film. The grids were rinsed briefly in 95 % ethanol, then stained in 95 % ethanol containing 0.01 % uranyl acetate for 1 min and rinsed again in 95 % ethanol. The grids were rotary shadowed by evaporation of platinum–palladium at an 8° angle under a 1×10^{-6} Torr (1 Torr = 1.33×10^2 Pa) vacuum. The HA fragments were examined and photographed in a Hitachi H-7000 electron microscope (Tokyo, Japan) operating at 25 kV. An electron graphics tracer (Landsdale, PA, U.S.A.) was used on EMs to obtain the end-to-end distance for HA molecules. The HA molecules were easily distinguishable from the DNA found in cellular samples, as the DNA was much wider. The magnification for each micrograph was used to calculate the actual molecular length in nm for each chain. The number of dimers per chain was determined by division of the length with 0.98 nm/dimer [14,15], and multiplication of this value by a value of 379/dimer yielded the M_r of the chain. The possibility of selective transfer for HA of different sizes was examined by subjecting HA of varying lengths to Sephacryl S-1000 chromatography. Recoveries varied by less than 20 % throughout the elution profile and were not related to M_r , indicating that selective transfer of HA of different sizes to the grids was not a factor.

RESULTS

Characterization of the HA produced by Swiss 3T3 fibroblasts

Log-phase Swiss 3T3 fibroblasts, maintained as described in the Experimental section, were incubated with [14 C]GlcNAc for 24 h. Media and cellular samples were processed, stored and subsequently analysed by Sepharose 4B chromatography. The resultant fractions were assayed for both 14 C incorporation and HA content. For both medium and cellular samples, HA and incorporated radiolabel were found almost exclusively in the V_0 (results not shown). Identification of the V_0 radioactivity as HA was confirmed by overnight digestion with hyaluronidase (from *S. hyalurolyticus*, 100 units) at 37 °C, followed by re-application

Figure 1 Electron micrograph of medium HA

Medium from cells in log phase was fractionated on a Sepharose 4B column and the V_0 peak containing the HA was prepared as described in the Experimental procedures section. The HA was visualized by rotary metal shadowing.

to the Sepharose 4B column. No counts above background were observed in the fractions associated with the V_0 , with the only observable peak eluting at the V_i of the column. It appears, therefore, that all the high M_r radioactivity is present as HA. Since virtually all of the HA was found in the V_0 , it would appear that only very large HA is released into the medium. While this would suggest an $M_r > 4 \times 10^6$ for proteins or dextrans, an HA molecule with an $M_r < 0.4 \times 10^6$ also occupies sufficient volume in solution to elute in the V_0 [16]. Another method was necessary to obtain a precise M_r value for the HA produced by Swiss 3T3 cells. Using a column with a larger exclusion volume would not ameliorate this situation, since chromatographic standards for HA are not commercially available and chromatographic selectivity curves for HA are only for HA of $M_r < 0.8 \times 10^6$ (product literature for Sephacryl S-1000, Pharmacia Fine Chemicals). We decided to take EMs of individual molecules of HA and to determine their M_r s by direct measurement of the molecular length, as described in the Experimental section. A typical EM of an HA molecule is shown in Figure 1. Since the HA samples are boiled in SDS before processing, there should be little protein interaction to stabilize the HA in any condensed conformation. Using 0.98 nm as the length per GlcNAc-GlcA dimer for HA in solution [14,15], an average M_r of $5.2 \times 10^6 \pm 1.3 \times 10^6$ ($n = 25$) was observed for HA derived from culture media. Applying this methodology to cellular samples yielded an average M_r of $1.8 \times 10^6 \pm 1.9 \times 10^6$ ($n = 53$) for cell-associated HA.

Figure 2 Time course of HA synthesis in isolated membranes

Membrane protein (200 μ g) was incubated in the presence of UDP-GlcNAc and [14 C]UDP-GlcA (1 μ Ci), as described in the Experimental section. The 14 C content of the fractions was determined for incubations of 1 (■), 5 (○), 10 (▲), 15 (□) and 60 (●) min.

HA synthetase activity of isolated membrane preparations

The HA synthetase activity of isolated membranes was measured using an assay system similar to that used by Ng and Schwartz [17]. A small amount of membrane protein, typically 100–200 μ g, was incubated at 37 °C in the presence of an excess of precursors, which contained 1 μ Ci of [14 C]UDP-GlcA label. Preliminary experiments using a 1 h incubation with log-cell membranes found that HA and incorporated radioactivity eluted in the Sepharose 4B V_0 . The M_r of HA in the membranes was determined by electron microscopy to be $0.14 \times 10^6 \pm 0.15 \times 10^6$ ($n = 72$). In order to evaluate the effect of incubation time on incorporation of label, 200 μ g of membrane protein from log cells was incubated for 1–60 min with the radiolabel-containing assay cocktail. Each of the samples was then applied to a Sephadex G-200 column and the fractions assayed for 14 C content. The change from Sepharose 4B was originally made to facilitate the detection of short HA chains that might be labelled at early time points, as seen by Mian [18]. Throughout the entire time course of 1–60 min, the only evidence of incorporated label was seen in the V_0 of the column (Figure 2). A sample of V_0 material was treated overnight with hyaluronidase and rechromatographed. The counts then eluted at V_t of the column, confirming the identity of the labelled material as HA. When the radioactive content of the V_0 peaks were totalled and compared with the time of incubation, the incorporation of label was proportional to the length of incubation. Throughout the range of protein quantities investigated (50–300 μ g), the observed 14 C incorporation was also determined to be proportional to the amount of membrane protein used in the assay.

Comparison of HA synthetic rates in intact log cells and in their isolated membranes

The ability of Sepharose 4B chromatography to quantify the amount of radiolabel incorporated as HA was used in an incubation time course to determine the time required to synthesize a complete HA chain. Two days after subculture, a new medium containing 10 μ Ci/flask of [14 C]GlcNAc was added to cells and growth allowed to continue for 24 h. The radioactive medium was removed and incubation continued for 0–8 h in the

Figure 3 Release of [14 C]HA from log cells

Log cells were grown for 24 h in medium containing [14 C]GlcNAc. Incubation was continued for 1–8 h in medium lacking [14 C]GlcNAc. The cell lysates from each time point were subjected to Sepharose 4B chromatography and the [14 C]HA determined from the total V_0 radioactivity. The [14 C]HA content at each time is expressed as a percentage of the 0 h [14 C]HA

presence of label-free medium. The cells were removed by treatment with trypsin, lysed by boiling in 1 % SDS for 3 min and the lysates stored at 4 °C for Sepharose 4B chromatography. Aliquots of fractions from the column runs were assayed for radioactivity and the 14 C content expressed as a percentage of the amount of V_0 label present in the cells isolated before introduction of label-free medium. Assuming uniform radiolabelling of the chains, the closer a chain is to completion, the more radioactivity it will contain. When incubation is continued in label-free medium, all chains are elongated and eventually released, but the radioactive content of individual chains should depend solely upon the amount of radioactivity incorporated during the labelling period, assuming that the precursor pools re-equilibrate rapidly upon removal of medium label. Given a uniform distribution of sizes of HA chains attached to the cell, the relationship between incubation time in label-free medium and remaining cellular [14 C]HA is described by the following relationship: $t/T = 1 - \sqrt{R}$, where T is the time required for the synthesis of a complete HA chain and R is the fraction of total initial (0 h) [14 C]HA remaining at any given time t , when $0 \leq t \leq T$. By measuring the remaining radioactivity at a given time, one can calculate T , the time required to synthesize a complete HA chain. As shown in Figure 3, at 1 h the remaining cellular radioactivity is only 0.59 of the 0 h value, decreasing to 0.39 after 2 h. These amounts correspond to values of 4 and 5 h respectively for the time required to synthesize a mature HA chain. In the event that some labelled precursor remains within the cell after the medium change, this would shorten these times somewhat, so they should be viewed initially as the upper limit for the time to make an HA chain *in vivo*. However, our data indicate that very little labelling occurs after the removal of label; by the 4 h time point, only 13 % more 14 C was found in the medium than was released from the cells, so the proposed synthesis time of 4–5 h should be close to the actual value.

In a related experiment, we determined the amount of HA attached to log-phase cells and the rate at which this HA is released into the medium. The results in Figure 4 (untreated cells) show that HA is released into the medium at a rate of 2.8 nmol HA dimer/h per 10^6 cells over the 8 h time course. The amount of HA associated with the cells was found to be 5.5 nmol dimer/ 10^6 cells. The cellular HA changed very little throughout the experiment, so it is reasonable to assume that nearly all of the HA synthesized was destined for export to the medium. Since these cells are continuously synthesizing HA, chains in all stages

Figure 4 Effect of cycloheximide on media HA synthesis of log cells

Log cells were grown for 1–8 h in media containing 10 $\mu\text{g}/\text{ml}$ cycloheximide. The HA content of the medium at each time was determined for cycloheximide-treated cells (■) and untreated control cells (○).

of completion should be equally represented on the cell surface. Chains that have been just initiated will be present, as will chains that are nearly completed, so that on average, the chains on the surface are halfway completed. The total HA content of these chains when they are all completed will, therefore, be twice the amount that they possess while still attached to the cell. Thus, the amount of HA released into the medium (M) in a given time (t) is equal to the fraction of the time required for completion of an entire chain that has elapsed ($= t/T$, where T is the time needed to synthesize an entire chain) at time t , times twice the amount of cell-associated HA (C); i.e. $M = t/T \times 2C$. Based on a rate of release of 2.8 nmol dimer/h per 10^6 cells, 2 h would be required for the 5.5 nmol of dimer of cell-associated HA per 10^6 cells to be released into the medium. Twice this value, which is 4 h, is the time we calculate to be required to make a complete HA chain. Given a $5.2 \times 10^6 M_r$ for mature HA, this represents an elongation rate of about 57 dimers/min *in vivo*.

Analysis of the HA contained in the membrane preparations was hampered by our inability to obtain HA sizes directly from gel filtration chromatography. As is evident from Figure 2, all of the HA present in the membranes is large enough to be excluded in Sephadex G-200 chromatography and the labelled precursor is incorporated into these chains. Thus the membrane HA in our system is unlike that obtained by Mausolf et al. [19] and Mian [18] in which the nascent HA was initially very small and the orderly change in size of incorporated label could be used to obtain a rate (dimers/min) of HA chain elongation. Since we could not follow the change in M_r with length of incubation, we determined the number of potential sites (i.e. HA chains) available for HA synthesis in the membrane preparation and then related this to the observed dimer synthesis. This approach allows us to calculate a lower limit for the rate of elongation, since, if not all of the HA in the assay is capable of sustaining synthesis, the calculated value will be lower than the true rate of HA synthesis. The number of HA chains available for elongation was obtained by taking the nmol of HA dimer present, converting it into the number of dimers by multiplication with Avogadro's number, followed by division with 369 dimers, which is the number of dimers present in a chain of M_r 0.14×10^6 . The number of newly synthesized dimers was calculated based on the incorporated radiolabel determined by Sephadex G-200 chromatography. Using log cells, the amount of HA present in an assay was about 0.45 nmol of HA dimer/mg of protein ($\approx 7.3 \times 10^{11}$ chains of M_r 0.14×10^6 per mg of protein), with a

Figure 5 Effect of cycloheximide on the HA synthetase activity of isolated membranes

Log cells were incubated for 2, 4 and 6 h in the presence of 10 $\mu\text{g}/\text{ml}$ cycloheximide before isolation of their membranes. The HA synthetase activity of the membranes was determined at each time as described in the Experimental section and is expressed as a percentage of the 0 h time point (■). The effect of cycloheximide treatment on intact cell synthesis of medium HA during the time period is shown for comparison (○).

typical HA synthetic rate of 0.15 nmol of dimer/h per mg of protein ($\approx 9.0 \times 10^{13}$ dimers/h per mg protein). Assuming all of the HA present is capable of elongation, the rate of chain extension would be 2 dimers per min.

Effect of cycloheximide on HA synthesis by intact log cells and on HA synthetase activity of membranes isolated from these cells

Previous investigations have determined that cells lose their ability to make HA when protein synthesis is prevented by agents such as 1–100 $\mu\text{g}/\text{ml}$ cycloheximide [18,20]. A concentration of 10 $\mu\text{g}/\text{ml}$ cycloheximide was found to terminate protein synthesis efficiently in our Swiss 3T3 cells. The effect of cycloheximide on HA synthesis was determined in logarithmically growing cells. The medium of log-phase cells was replaced with fresh medium containing 10 $\mu\text{g}/\text{ml}$ cycloheximide and growth was allowed to continue. Samples were drawn from the medium hourly for 8 h, made 1% in SDS, boiled for 3 min and stored at 4 $^{\circ}\text{C}$ for Sepharose 4B chromatography. Upon completion of the chromatography, the fractions from each sample were assayed for HA content. The amount of HA released into the medium is virtually identical after 1 h for the cycloheximide-treated and untreated cells (Figure 4). However, after 2–3 h exposure, the rate of HA synthesis of exposed cells drops to half that of untreated cells, and by 4 h of cycloheximide exposure, the total HA exported to the medium by cycloheximide-treated cells is only 50% of that of untreated cells. The decrease in synthetic rate of the treated cells was accompanied by a decrease in the amount of cell-associated HA. At the end of the 8 h cycloheximide incubation, exposed cells possessed only 33% of the cell-associated HA observed in untreated control cells.

We next examined the effect of cycloheximide treatment on the HA synthetase activity of membranes isolated from exposed cells. Cells were grown to log phase and their medium removed and replaced with new medium. Cycloheximide was added and the cells incubated in its presence for 0, 2, 4 and 6 h. Figure 5 shows the decline in HA synthetase activity in membranes of cells incubated with cycloheximide; results from intact cells treated with cycloheximide (from data given in Figure 4) are included for comparison. Within 4 h of cycloheximide treatment, the mem-

Figure 6 Effect of cycloheximide on medium HA synthesis and cell-associated HA content of serum-stimulated quiescent cells

Quiescent cells were stimulated by addition of new medium containing 10% CS. Cycloheximide exposure (10 μ g/ml) was initiated by its addition at the 0 h of serum stimulation. The HA content of (■) the medium and (b) the cell lysates was determined at each time for untreated cells (■) and cells exposed to cycloheximide (○).

brane HA synthetase activity decreases to 37% of untreated levels, dropping slightly at 6 h exposure. This decline in HA synthetase activity of the isolated membranes follows closely the decrease in HA release into the medium observed in cycloheximide-treated cells.

HA synthetic rates in quiescent cells and of membranes isolated from quiescent cells

HA synthesis was determined in quiescent cells and in quiescent cells stimulated by the addition of media containing 10% CS (Figure 6a, untreated cells). HA synthesis during the first 2 h following serum stimulation was 0.56 nmol dimer/h per 10⁶ cells, which is 20% of the rate observed in log cells. At 4 h of

stimulation, however, synthesis increases dramatically. From 4 to 16 h, synthesis of HA proceeds at a relatively constant rate of 2.4 nmol dimer/h per 10⁶ cells, which is 91% of the rate observed for log cells. During the 16 h time course, the amount of cell-associated HA increases nearly 3-fold (Figure 6b, untreated cells), from 1 to 3 nmol dimer/10⁶ cells, which are 20 and 56% of log-cell levels respectively.

We next determined the HA synthetase activity of membranes isolated from log, quiescent and serum-stimulated quiescent cells (16 h after serum stimulation). This was performed by assaying 200 μ g of membrane protein, as described in the Experimental section. The HA synthetase activity of quiescent cells was 27% of log-cell levels. Serum-stimulation increased the HA synthetase activity of quiescent cell membranes to 55% of that of log-cell membranes. These results are presented in Table 1, which provides a comparison of the relative synthetic rates, cell-associated HA contents and membrane HA synthetase activities of Swiss 3T3 fibroblasts subjected to four different experimental growth conditions.

Effect of cycloheximide on cell-associated HA levels and HA synthetase activity of serum-stimulated quiescent cells

We next determined the effect of cycloheximide (10 μ g/ml) on the increases in HA synthesis and cell-associated HA that follow serum stimulation of quiescent 3T3 fibroblasts. Cells were grown to quiescence, as described in the Experimental section, and stimulated by the addition of fresh medium containing 10% CS. Cells were exposed to cycloheximide for 0–8 h following serum stimulation. At hourly intervals, cells and media were made 1% in SDS, boiled for 3 min and stored at 4 °C for later analysis. At the end of the experiment, cellular and media samples were applied to a Sephadex G-25 column and the fractions analysed for HA content. The results for medium HA are shown in Figure 6(a) and for cell-associated HA in Figure 6(b). As stated earlier, the untreated cells exhibited slow synthesis of medium HA for the first 4 h, followed by a sustained burst of synthetic activity for the remaining 12 h. The cycloheximide-treated cells also demonstrated low levels of HA synthesis, comparable to that of untreated cells at early time points. In contrast to the untreated cells, however, the rate of HA synthesis remained depressed. The levels of HA that remained associated with the cells under the various conditions are shown in Figure 6(b). The amount of HA found attached to the cells decreased similarly during the first 2 h after the initial medium replacement for both cycloheximide-

Table 1 Effect of proliferative ability on the HA content and HA synthetic ability of Swiss 3T3 cells and their membranes.

	Cell-associated HA*	HA release in medium*	HA synthetase activity of isolated membranes*
Log cells (%)	100 (5.5 nmol dimer/10 ⁶ cells)	100 (2.8 nmol dimer/h per 10 ⁶ cells)	100 (0.15 nmol dimer/h per μ g of protein)
Log cells, exposed to cycloheximide (%)†	33	18	34
Quiescent cells (%)	20	20	25
Quiescent cells, serum-stimulated‡ (%)	56	91	51

* For each of these cell populations, the cellular HA content, rate of HA release into the culture medium and HA synthetase activity of their isolated membranes was determined as described in the Experimental section.

† Cycloheximide-treated cells were exposed for 8 h before use.

‡ Stimulation of quiescent cells was performed by incubation with 10% CS for 16 h.

treated and untreated cells. However, whereas the levels of cellular HA in untreated cells rebounded and exceeded pre-experimental values, the cycloheximide-treated cells continued to lose HA from their surfaces before stabilizing at a level of about 40 % of their original content. Untreated cells continued to add to the HA associated with their surface until the 11–12 h time points, eventually stabilizing at a level approx. 300 % of original levels.

DISCUSSION

We determined the M_r of HA in the medium and the HA associated with the cell surface of Swiss 3T3 fibroblasts to be 5.2×10^6 and 1.8×10^6 respectively. These values fall well within the mainstream of sizes reported for HA. For example, HA from rooster comb has an M_r of 1.2×10^6 and HAs from human and bovine synovial fluid possess M_r s of 6×10^6 and 12×10^6 respectively [21]. Given the amount and sizes of HA that we determined in the medium, and that which is cell-associated, we derived the following quantities that describe the physical state of HA at the level of a single cell. We determined that a cell in log phase contains 2.8×10^{-15} nmol of HA dimer on its surface, which corresponds to 680000 molecules of HA with an average M_r of 1.8×10^6 . We also observed the rate of HA release to be 5.5×10^{-15} nmol of HA dimer/h per cell, which, for an M_r of 5.2×10^6 , indicates a loss of 120000 HA chains each hour from each cell. Furthermore, approx. 4 h is required by intact Swiss 3T3 fibroblasts for the synthesis of a completed HA chain of M_r 5.2×10^6 . Given the published volume for murine Swiss 3T3 fibroblasts of $5 \mu\text{l}/10^6$ cells [22], values of 5×10^{12} nm³ for the volume, 10600 nm for the radius and 1.4×10^9 nm² for the surface area of a single (spherical) cell can be derived. The surface of a log-phase cell must accommodate 680000 HA molecules, which means that the area on the cell surface occupied by a single chain would be 2075 nm², if there is no chain overlap. Using 27×10^6 nm³ as the volume occupied by a HA molecule of M_r 8×10^6 in random coil conformation [21], we calculate the volume of a cellular HA chain of M_r 8×10^6 to be 6.1×10^6 nm³. Since the area occupied by the HA chain on the cell surface is constrained to be 2075 nm² (45.5×45.5 nm), we calculate the dimensions for an average (M_r 1.8×10^6) HA chain to be approx. $45.5 \times 45.5 \times 2930$ nm. Based on a 0.98 nm length per dimer [14], a chain of M_r 1.8×10^6 has a length of 4650 nm fully extended. This indicates that nascent HA chains extend, for the most part, directly away from the cell. It is clear that the halo of HA that surrounds a cell occupies a considerable volume, which, if the HA is in a totally non-overlapping random coil conformation, will be nearly equal to the volume occupied by the interior of the cell. Given the molecular sieve properties reported for HA [6,23], the size of the HA halo could exert a considerable influence on the cellular entry and exit of macromolecules. Also, this effect may be exaggerated with proteins that bind HA, such as hyaluronidases, whose penetration may be slowed due to the high ligand concentration at the halo periphery.

The M_r of HA in our membrane preparation (0.14×10^6) is substantially less than the M_r of the cellular HA (1.8×10^6) from which it is isolated. Thus it appears that we are selectively isolating the smallest of the cellular HA chains. Previous work by Philipson et al. showed that pre-treatment of cells with hyaluronidase results in an increase in the HA synthetase activity of isolated membranes [24]. It is unlikely that hyaluronidase acts directly on the synthetase to increase its activity since they found that direct treatment of the membranes with hyaluronidase did not increase HA synthetase activity [24]. It is probable that the

increase in enzymic activity observed in cells treated with hyaluronidase is due to an increase in the recovery of enzyme during membrane isolation. Given the fact that our membranes retain only 0.8 % of the cellular HA from which they are derived, we calculate that preferential isolation of the shortest chains would yield membrane HA of M_r 0.47×10^6 , while simply shearing of the outermost part of all cellular HA chains would predict a membrane HA M_r of 0.02×10^6 . Since our observed membrane HA M_r of 0.14×10^6 is midway between these values, it appears that both processes are at work. Pretreatment with hyaluronidase results in the shortening of cellular HA, which should reduce the amount of shear experienced by cellular HA chains during sonication. Since, to a significant extent, we are preferentially isolating the shorter chains, this would increase the number of cellular HA chains recovered during membrane isolation and, also, the number of HA synthetase molecules that are isolated while still bound to nascent HA chains. Moreover, we found that subjecting isolated membranes to exhaustive hyaluronidase digestion before assaying for HA synthesis yielded HA synthetase activities that were < 20 % of control levels (results not shown). Thus it appears that preserving the bond between HA synthetase and its chain, perhaps an HA primer of a critical minimum length, is necessary for the synthetase to polymerize HA.

Pretreatment of log-phase cells with cycloheximide for approx. 3 h reduces the HA synthetase activity of membranes from log cells by half (Figure 5). Also, the rate of HA synthesis dropped to half that of control cells and total HA production was reduced to half that of control levels by 4 h (Figures 4 and 5). We also examined the effect of cycloheximide on the pattern of HA release by quiescent cells (Figure 6). During the first 2 h after serum stimulation, cycloheximide-treated and untreated cells were virtually indistinguishable in their release of HA into the medium and in their loss of cell-associated HA. Moreover, while the levels of HA released and cell-associated HA remained depressed in treated cells, both quantities increased dramatically within 2–3 h of serum stimulation when protein synthesis was not inhibited. These results suggest that inhibition of protein synthesis interferes with the initiation of new HA chains but does not alter elongation and release of existing chains.

The biological lifetime of HA synthetase (4–6 h; twice the half-life) is similar to the amount of time (4–5 h) required to synthesize a complete HA chain (calculated from results presented in Figures 3 and 4). Both of these values were obtained by techniques independent of EM size determinations and, taken with the results of the EM size determinations, suggest that HA synthetase is inactivated upon completion of the HA chain it is synthesizing. One mechanism for termination of HA synthetase activity that is consistent with the data presented is for HA synthetase to remain bound to an HA chain even after its completion, followed by release from the cell along with the completed chain. If this hypothesis is correct, there should be a correlation between the amount of HA attached to the cell exterior, the amount of HA synthetase activity and the HA synthetic capacity of the cell. We examined the amount of cellular HA associated with log cells, log cells exposed to cycloheximide, quiescent cells and quiescent cells stimulated by serum addition, and compared these values to their HA synthetic capacities. As seen in Table 1, the amount of cellular HA present on the surfaces of these various cell populations is followed closely by the ability of their membranes to synthesize HA. There is also a strong correlation between the amount of cell-associated HA and the rate of HA release into the medium by intact cells. Relevant data are found in a recent publication by Mausolf et al. [19]. They reported that HA synthetase is shed from *Streptococci* along with HA. Included in

the data were values of HA ($\mu\text{g/ml}$) and HA synthetase ($\mu\text{g/ml}$) found in the medium during an 8 h time course (from Figure 1, [19]). Values of approx. $0.5 \mu\text{g}$ of HA synthetase/ml of medium and $34 \mu\text{g}$ of HA/ml of medium were given for the 8 h time point. Although no value was given for the size of the HA released into the culture medium, the HA of protoplast membranes exhibited a mean $M_r > 1 \times 10^6$ (from Figure 9, [19], 120 min time point) [19], with a significant amount eluting in the V_0 of a Sephacryl S-1000 column. The elution pattern they reported is similar to what we observed when fractionating a sample containing HA of mean M_r 3.8×10^6 on a Sephacryl S-1000 column (results not shown). Using 52000 as the M_r for the soluble synthetase [19] and $(3-4) \times 10^6$ to approximate the $M_{r,s}$ of their mature HA chains, values of 9.6 pmol/ml HA synthetase and $8.5-11.3 \text{ pmol/ml}$ mature HA are derived. The nearly one to one ratio of HA synthetase to HA chains strongly supports the idea that HA synthetase is lost from the cell along with the completed HA chain.

Termination of HA synthetase activity by release of the enzyme (or critical subunit) along with its solitary product would be a unique mechanism for the cellular regulation of HA synthesis. This is unlike most enzymes, whose activity is lost due to proteolytic degradation after the completion of many products. Initiation of HA synthesis would occur by the synthesis of new enzyme, or subunit, perhaps with an HA primer already attached, followed by export to the cell surface. The new complex would be responsible for the synthesis of one complete HA chain.

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