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Hyaluronate is synthesized at plasma membranes

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The hybrid cell B6 line, which synthesizes large amounts of hyaluronate as the predominant glycosaminoglycan, was grown in the presence of [^{3}H]glucosamine. The [^{3}H]hyaluronate has a high molecular weight and was excluded by Sephacryl S-1000. After disruption of the cells the [^{3}H]hyaluronate could further be elongated by incubation with UDP-GlcNAc and UDP-[^{14}C]GlcA, yielding a hybrid molecule of hyaluronate labelled with [^{3}H]GlcNAc and [^{14}C]GlcA. Treatment of the cells with hyaluronidase before disruption eliminated the large [^{3}H]hyaluronate and elongation of nascent chains *in vitro* commenced from low-molecular-weight chains. Thus nascent hyaluronate chains were degraded extracellularly by hyaluronidase and were therefore synthesized at the inner side of plasma membranes and extruded to the cell surface.

Hyaluronate has recently been shown to be synthesized at the reducing end by alternate transfer of UDP-hyaluronate to the substrates UDP-GlcNAc and UDP-GlcA (Prehm, 1983*a*,*b*). This mechanism differs from the synthesis of glycopeptides and proteoglycans, which are elongated at the non-reducing end on lipid carriers or on protein primers, respectively. Hyaluronate is the only glycosaminoglycan to be produced both by mammalian cells and bacteria. Since bacteria do not possess intracellular organelles, they cannot synthesize hyaluronate in Golgi vesicles and cannot secrete it by fusion of secretory vesicles with plasma membranes. Therefore it seemed likely that hyaluronate is also secreted in mammalian cells by another mechanism.

Materials and methods

Materials

B6 cells were obtained from Dr. I. Hilwig, Hoechst AG, Frankfurt, Germany. The cells were grown in Dulbecco's modified Eagles medium supplemented with streptomycin/penicillin (100units/ml), kanamycin (100μg/ml) and 10% (v/v) foetal bovine serum. Hyaluronidase (EC 3.2.1.35) from *Streptomyces hyalurolyticus* (proteinase-free; 2000 NF units/mg) was from Miles; [^{3}H]-glucosamine (24.8Ci/mmol) and UDP-D-[^{14}C]-GlcA (264mCi/mmol) from Amersham International; Sephacryl S-1000 from Pharmacia.

Abbreviation used: SDS, sodium dodecyl sulphate.

Substrate A for hyaluronate synthesis *in vitro* was 8μM-UDP-[^{14}C]GlcA, 166μM-UDP-GlcNAc, 10mM-$MgCl_2$ and 4mM-dithiothreitol. Hyaluronate synthesis was measured as described by Prehm (1980).

Elongation in vitro of metabolically labelled hyaluronate

B6 cells were grown for 2 days to a cell density of 4×10^5 cells/ml in 20ml of medium. The medium was supplemented with 2ml of foetal bovine serum and 50μCi of [^{3}H]glucosamine. After 4h of incubation the cells were centrifuged off for 5min at 1000*g*, washed twice with 10ml of phosphate-buffered saline at 4°C and divided into three parts.

(*a*) The cell sediment was suspended in 100μl of hyaluronate solution (10mg/ml). One-half of this solution was added to sample (*c*). The rest was twice frozen and thawed, supplemented with substrate A and incubated for 30min at 37°C. After addition of 10μl of 10% (w/v) SDS the suspension was boiled for 3min and filtered on a Sephacryl S-1000 column (0.8cm × 96cm) with phosphate-buffered saline containing 0.1% SDS as eluent at a flow rate of 5.6ml/h. Fractions (0.7ml) were collected and 0.5ml aliquots were withdrawn for determination of the radioactivity.

(*b*) The cell pellet was suspended in 1ml of phosphate-buffered saline containing 5NF units of bacterial hyaluronidase for 30min at 37°C. The cells were washed twice with 15ml of cold phosphate-buffered saline, suspended in 100μl of

hyaluronate solution (10mg/ml) and processed further as sample (*a*).

(*c*) The cell pellet was treated in the same way as described for sample (*b*) except that one-half of sample (*a*) was added after washing with phosphate-buffered saline.

Introduction of [^3H]hyaluronate into the cell, hyaluronidase digestion and elongation of hyaluronate in vitro

B6 cells (6.3×10^6) grown for 2 days were washed twice with phosphate-buffered saline and incubated for 10min at 37°C with a mixture of 0.3ml of [^{3}H]hyaluronate (4.6×10^5 c.p.m.), 0.5ml of 1M-sucrose and 0.2ml of 50% (v/v) poly(ethylene glycol) 1000 in Dulbecco's modified Eagles medium. The mixture was centrifuged for 3min at 1000*g* and the cell pellet was incubated with a mixture of 6ml of Dulbecco's modified Eagles medium and 4ml of water and again sedimented for 3min at 1000*g*. The cell pellet was once again subjected to this incubation sequence and then incubated with 3NF units of hyaluronidase in 1ml of phosphate-buffered saline for 30min at 37°C. The cells were sedimented for 3min at 1000*g*, washed twice with 15ml of phosphate-buffered saline, suspended in 100μl of unlabelled hyaluronate (1mg) and 100μl of substrate A, disrupted by freezing and thawing, and incubated for 30min at 37°C. After addition of 10μl of 10% (w/v) SDS the suspension was boiled for 3min and filtered on Sephacryl S-1000.

Results

Intact B6 cells were mixed with substrate A in phosphate-buffered saline.One-half was incubated directly, the other half was frozen and thawed to disrupt the cells and hyaluronate synthesis was measured as described by Prehm (1980). The hyaluronate synthase activity in intact cells was 10% of that in disrupted cells. Because UDP-GlcNAc and UDP-GlcA are not taken up by cells, hyaluronate synthase must be localized intracellularly.

The following experiments were designed to distinguish between an intracellular or extracellular growth of nascent chains.

B6 cells were labelled with [^{3}H]glucosamine and supplemented with serum, which is known to stimulate hyaluronate synthesis several-fold (Tomida *et al.*, 1975). After disruption of the cells, hyaluronate synthesis was started by addition of substrate A for 30min and chain length was determined by gel filtration on Sephacryl S-1000. Fig. 1(*a*) shows the elution profile of the ^{3}H- and ^{14}C-labelled material. The radioactivity eluting in fractions 20–60 was hyaluronate, because it was

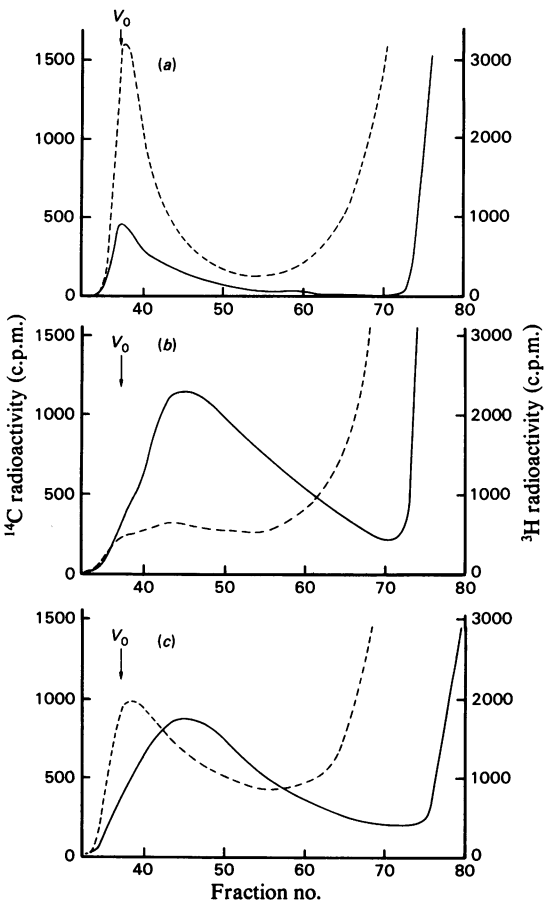

Fig. 1. *Elongation of metabolically labelled [^3H]hyaluronate by UDP-GlNAc and UDP-[^{14}C]GlcA in vitro*
The Figure shows gel fitration on Sephacryl S-1000 of samples (*a*) without hyaluronidase treatment, (*b*) with hyaluronidase treatment of intact cells, and (*c*) a mixture of hyaluronidase-treated and untreated cells. Details were described in the Materials and methods section. -----, ^{3}H radioactivity; ——, ^{14}C radioactivity. Low-molecular-weight fractions have been omitted for simplicity.

susceptible to bacterial hyaluronidase. The majority of the hyaluronate was eluted in the excluded volume with some tailing into the included volume. Thus pre-existing high-molecular-weight [^{3}H]-hyaluronate seemed to be elongated by broken cells with UDP-[^{14}C]GlcA and UDP-GlcNAc.

Extracellular digestion of hyaluronate on B6 cells and subsequent elongation *in vitro* should provide direct evidence whether cells secrete hyaluronate as completed chains or as nascent chains.

When [^{3}H]GlcNAc-labelled cells were incubated with bacterial hyaluronidase and then with substrate A for hyaluronate synthesis *in vitro*, the

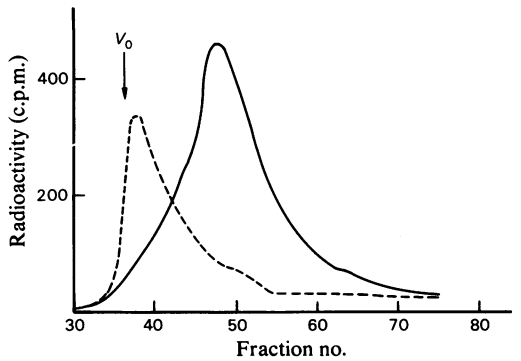

Fig. 2. *Protection of internalized [^{3}H]hyaluronate against extracellular hyaluronidase*

Details were described in the Materials and methods section. -----, ^{3}H radioactivity; ——, ^{14}C radioactivity. Low-molecular-weight fractions have been omitted for simplicity.

amount of [^{3}H]hyaluronate was greatly reduced and [^{14}C]hyaluronate was eluted in the included volume (Fig. 1*b*). The cells remained fully viable after hyaluronidase treatment, as indicated by Trypan Blue exclusion. The same molecular weight of [^{14}C]hyaluronate was obtained when isolated membranes from B6 cells were treated with hyaluronidase and incubated with substrate A (results not shown). The [^{14}C]hyaluronate must have been elongated from short nascent hyaluronate chains which had been cut by hyaluronidase. Therefore [^{3}H]hyaluronate was accessible to extracellular hyaluronidase and could be elongated by broken cells.

The following experiment demonstrated that no residual hyaluronidase was left in the incubation mixture which could account for partially degraded hyaluronate. Untreated and hyaluronidase-digested cells were mixed, incubated with substrate A and the mixture was again chromatographed on Sephacryl S-1000 (Fig. 1*c*). The [^{3}H]hyaluronate was eluted undegraded in the excluded volume and the [^{14}C]hyaluronate again in the included fractions. The radioactivity in fractions 34–55 was hyaluronate, because it was degraded by bacterial hyaluronidase.

To exclude the possibility that hyaluronidase was internalized by cells and digested to nascent chains intracellularly, [^{3}H]hyaluronate of high molecular weight was introduced into the cell by osmotic lysis of pinocytotic vesicles (Okada & Rechsteiner, 1982). This method has been shown to transfer macromolecules into the cytosol. The cells were then treated with hyaluronidase, disrupted, and incubated with substrate A for 30min. The molecular weights of [^{3}H]- and [^{14}C]hyaluronates were compared by gel filtration on Sephacryl S-1000 (Fig. 2). The internalized [^{3}H]hyaluronate was undegraded and the [^{14}C]hyaluronate had a size originating from short nascent chains.

Discussion

Most glycosaminoglycans of mammalian cells are synthesized in the Golgi. Hyaluronate has also been claimed to be synthesized in this organelle, as based on electron microscopic autoradiography of cells labelled with [^{3}H]glucosamine (Barland *et al.*, 1967). However, metabolic labelling with [^{3}H]-glucosamine is not a reliable marker for hyaluronate, since it will be incorporated into many other glycoproteins and proteoglycans. Some doubts arose that the Golgi was the site of hyaluronate synthesis, when von Figura *et al.* (1973) found that proteoglycans and hyaluronate used different precursor pools of UDP-sugars. Mitchell & Hardingham (1982) substantiated this uncertainty, because they could not inhibit hyaluronate synthesis by monensin, which interferes with secretion in trans-Golgi vesicles. Synthesis of hyaluronate did not require a protein primer (Sugahara *et al.*, 1979; Mason *et al.*, 1982; Prehm, 1983*a*,*b*) or lipid intermediates (Ishimoto & Strominger, 1967). It was not inhibited by cycloheximide (Mapleson & Buchwald, 1981), xylosides (Hopwood & Dorfman, 1977) or tunicamycin (Hart & Lennarz, 1978). Hyaluronate was synthesized at the reducing end by alternate transfer of the chains to the substrate UDP-GlcNAc and UDP-GlcA. Chains can be initiated by the substrates themselves. Synthesis occured at membranes and was abolished by detergents (Prehm, 1983*a*,*b*).

The hybrid cell B6 derived from mouse mammary carcinoma and a Chinese hamster lung cell line (Koyama *et al.*, 1970; Koyama & Ono, 1970) was chosen for this study, because it produced large amounts of hyaluronate as the predominant glycosaminoglycan. Hyaluronate could efficiently be labelled with [^{3}H]glucosamine. It was poorly synthesized from UDP-GlcNAc and UDP-[^{14}C]-GlcA by intact cells, because nucleotide sugars do not penetrate the plasma membrane. However, when cells were disrupted by freezing and thawing, these precursors were incorporated into hyaluronate.

When B6 cells were metabolically labelled with [^{3}H]glucosamine and subsequently with UDP-[^{14}C]GlcA *in vitro*, hybrid molecules of hyaluronate were obtained labelled with [^{3}H]GlcNAc and [^{14}C]GlcA. Digestion of extracellular hyaluronate eliminated the large [^{3}H]hyaluronate and elongation *in vitro* produced ^{14}C-labelled chains of the same size as obtained from isolated hyaluronidase-treated membranes. Therefore nascent hyaluron-

ate of intact cells was susceptible to extracellular hyaluronidase.

These results are compatible with the notion that hyaluronate is synthesized inside the cell at plasma membranes and extruded to the extracellular matrix.

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