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Commentary

Does DG42 synthesize hyaluronan or chitin?: A controversy about oligosaccharides in vertebrate development

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Science generally progresses in slow but deliberate increments, which are punctuated by major advances in concept or fact. However, the latter are rare and not infrequently go unrecognized when they first occur. Another event that can add spice to a field, and attract the attention of scientists from outside the discipline, is a genuine controversy. One such controversy is presented by two reports in this issue of the Proceedings (1, 2). To be asked to referee such a controversy is an interesting but difficult task, since both groups have significant data to back their claims.

The story begins in 1983, when Igor Dawid and colleagues (1) reported the isolation of several genes that are Differentially expressed at Gastrulation (DG) in embryos of the frog, *Xenopus laevis* (3). One of these, the endoderm-specific DG42, is expressed in a short window during embryogenesis, being first detected after the midblastula stage, peaking at late gastrula, and decaying by the end of neuralation (4–6). Appropriate probes were used to show that the messenger RNA and predicted protein product move in a wave or gradient through the embryo, with the last remnants seen in the ventral regions of the gut at the tailbud stage. For a while thereafter, DG42 remained an interesting gene in search of a function. As often happens, the first clues came from unexpected sequence homology information. When it was first cloned, DG42 showed no obvious homologies to any previously known protein or gene. Subsequently, some similarities were found with fungal chitin synthases (7) and with the rhizobium NodC gene that is known to synthesize chitin oligomers (8–10).

What is chitin? It is a repeating β1-4-linked homopolymer of the monosaccharide GlcNAc (see Fig. 1) that is one of the most widespread and abundant molecules in the biosphere, providing, for example, a major component of the cell walls of fungi and the shells of crustaceans and arthropods (11, 12). This important structural role for the extended polysaccharide may seem of little relevance to vertebrate development. However, shorter oligomers of the same repeating sequence are known to be soluble "oligosaccharins," mediating short range hormonal responses between *Rhizobium* bacteria and leguminous plants during the process of nitrogen-fixing root-nodule formation (13–15). Indeed, complex structural variations on the theme of the basic chitin backbone are well known to mediate a variety of specific interactions between bacteria and plants (for some examples, see refs. 16–20).

Intrigued by these homologies, Semino and Robbins (21) then showed that when generated in an *in vitro* transcription/translation system, the DG42 gene product was capable of synthesizing both short chitin oligomers and some larger products. The required sugar nucleotide donor was UDP-GlcNAc; the products had the correct chromatographic properties, and they were degraded appropriately by a bacterial chitinase. Thus, DG42 was proposed to be the first recognized vertebrate chitooligosaccharide synthase (21). However, another interesting homology had also appeared between DG42 and the *hasA* gene of *Streptococci* (22, 23). The latter is responsible for the synthesis of another repeating polymer of sugars called hyaluronan.

What is hyaluronan? It is a polymer consisting of alternating units of β1-4-linked GlcNAc and β1-3-linked glucuronic acid (GlcA, see Fig. 1). At first glance, these may seem to be very similar structures. Indeed, the linkages are very similar, and the donor nucleotides for both units are based on UDP (UDP-GlcNAc and UDP-GlcA). However, the similarity ends there (24, 25). Partly by virtue of its carboxylate groups, hyaluronan has physical properties that are almost diametrically opposite to those of chitin, being capable of retaining large amounts of water to form a gel. Furthermore, unlike chitin, hyaluronan expression is primarily reported in vertebrates, and in a few pathogenic bacteria such as group A and C *Streptococci* (24, 25).

In view of these homologies, Semino and Robbins had also checked to see if the DG42 protein had hyaluronan synthase activity *in vitro*, but did not find any (21). This seemed to settle the issue that the DG42 gene product was primarily a chitin synthase. Enter the new study of Meyer and Kreil (1), which shows that rabbit kidney and human osteosarcoma cells induced to express the DG42 gene with a vaccinia virus system synthesize increased amounts of hyaluronan. Lysates and membranes from such transfected cells showed markedly increased hyaluronan synthase activity, which required the addition of both UDP-GlcNAc and UDP-GlcA donors. The product of the reaction was sensitive to hyaluronidases, but not to chitinases, and appropriate controls showed that the overexpression of hyaluronan synthesis was clearly related to DG42 expression (1). These authors conclude that their results are at variance with the earlier report of Semino and Robbins (21). Meanwhile, the latter group have an update to their story that is also published in this issue (2). They now show that DG42 homologues and their protein products are expressed in early embryos of zebrafish and mouse during the gastrula–early neuralation stages, and that chitin-oligosaccharide synthesis can be detected in extracts from these sources as well. Furthermore, this activity was immunoprecipitated by a DG42-specific antibody (4) provided by Dawid. Also, overexpression of DG42 in a different cell type (mouse 3T3 cells) gives the synthesis of chitooligosaccharides, but no increase in background levels of hyaluronan synthesis. Finally, these authors show a physical separation of chitin synthase activity from most (but not all) of the hyaluronan synthase activity in embryo extracts (2).

How can one reconcile the findings of the two studies and determine the true role of DG42? Semino *et al.* do make one preliminary attempt to do so (2). They state that commercial preparations of hyaluronan have chitin oligomers at their reducing end core region (further details are evidently to be published elsewhere). They suggest that DG42 might function to produce chitin oligomers that act as templates for hyaluronan synthesis (see Fig. 1). In this regard, it is interesting that Meyer and Kreil note a requirement for high concentrations of UDP-GlcNAc in their reactions (1).

To consider this possibility further, let us review what is known about hyaluronan synthesis in vertebrate systems. The biosynthesis of this polysaccharide is peculiar, in that it follows a route different from that taken by most other molecules

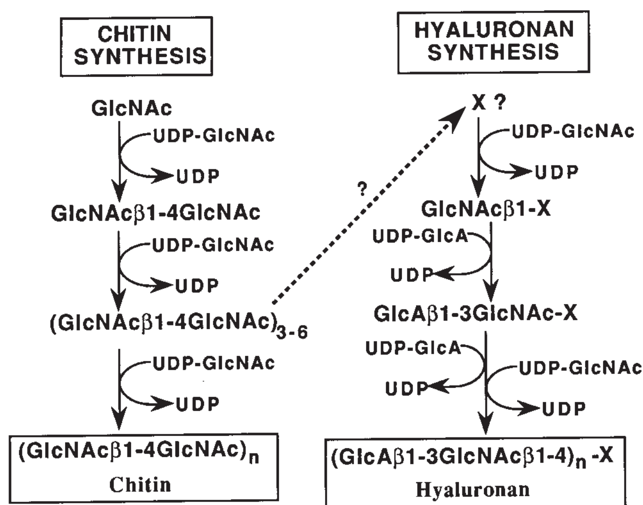

FIG. 1. Hyaluronan and chitin—similarities and differences. The known and proposed biosynthetic pathways for hyaluronan and chitin are shown, highlighting the similarities and differences. The X indicates the currently unknown primer for hyaluronan synthase action. The dotted line indicates the possibility raised here that small chitin oligosaccharides might act as primers for hyaluronan synthesis.

secreted by vertebrate cells. The great majority of secreted glycoconjugates (oligosaccharide-bearing macromolecules) are exported by cells via the endoplasmic reticulum–Golgi–plasmalemma pathway, wherein a complex machinery brings together sugar nucleotides, glycosyltransferases, and acceptors in a sophisticated assembly line system coupled with vectorial transport toward the cell surface (26, 27). In contrast, hyaluronan appears to be synthesized at or below the cell membrane and extruded directly at the cell surface, at the rate of about 1 residue per second (28–35). Unlike the bacterial enzyme, the mammalian hyaluronan synthase(s) have proven intractable to purification and characterization. Despite the efforts of several labs, the picture that emerges of the vertebrate hyaluronan synthase is confusing and even controversial in some respects (28–35). For example, the following questions remain unanswered. How many gene products (polypeptides) make up the hyaluronan synthase complex? Where exactly is it located? How are the sugar nucleotides provided to the enzyme? Does synthesis proceed in a processive manner, as suggested by some? Does the polymer grow from reducing end or the nonreducing end? If the latter, what is the primer for synthesis?

For many other polysaccharides, the importance of primers in the initiation of synthesis is clear (36). For example, the synthesis of chondroitin sulfate and heparan sulfate require the production of a xylose-linked tetrasaccharide core structure attached to the proteoglycan core protein (36). Likewise, the production of polysialic acids in vertebrate systems requires a sialylated lactosamine core that can be carried in a variety of glycoconjugate types (37). As shown in Fig. 1, the initiation of chitin production requires addition of the simplest core, a residue of GlcNAc (11, 12, 21). In contrast, remarkably little is known about how hyaluronan synthesis is primed. Indeed, the standard assay for hyaluronan synthesis does not involve the addition of any primer, implying either that it is not required or that endogenous primers are present in the extracts used. Semino *et al.* mention an indirect piece of evidence suggesting that the latter is the case—treatment of the embryo extracts with a chitinase markedly reduced subsequent detection of hyaluronan synthase activity (2). In contrast, prior digestion of the extract with hyaluronidase did not reduce enzymatic activity. On the other hand, neither group tried to use exogenous hyaluronan as a primer.

Semino and Robbins (21) have clearly shown that the DG42 gene product has chitin synthase activity *in vitro*. However, Meyer and Kreil (1) point out that *in vitro* translation might give a misfolded protein with aberrant functional activity. This is plausible, since the two reactions in question (chitin synthesis and hyaluronan synthesis) are very similar with regard to the use of UDP-sugar nucleotides and the generation of β1-(3)4 linkages. Meyer and Kreil also state that their product is not sensitive to chitinase (1). However, this interpretation is based on the fact that digestion with chitinase leaves being high molecular weight hyaluronan at the origin of the paper chromatography. If a short chitin core did exist, its cleavage products would have been small and represented by a small fraction of the radioactivity. The authors do not report that they checked for any small neutral fragments that might have migrated forward on the chromatogram. Indeed, if a short core region of GlcNAc residues were present, this would represent a very small fraction of the total label in the hyaluronan product and might easily be missed.

Meyer and Kreil show that the activity of hyaluronan synthase generally correlates with DG42 expression in the *Xenopus* embryo (1). However, as Semino *et al.* point out, hyaluronan is clearly expressed under many circumstances in the adult animal, where DG42 is apparently not (2). Thus, if they are correct about hyaluronan synthesis requiring an chitooligosaccharide primer, some other gene product must produce this core region in the adult.

Another possible argument against the hyaluronan synthase being encoded by DG42 is that this would require that two distinct reactions (transfer of GlcNAc and GlcA) be mediated by the same enzyme. However, there are some prior examples in which one enzyme synthesizes more than one oligosaccharide linkage (e.g., the polymerization of heparan sulfate) (38). Alternatively, DG42 might be only one component of a complex of polypeptides that direct hyaluronan synthesis. In this regard, it is interesting that Dawid and colleagues had earlier commented (4) that DG42 is an intracellular protein located at the periphery of the cell—this would be exactly the location expected for a hyaluronan synthase.

Where does all this leave us with regard to the controversy? There is no clear-cut answer. In the opinion of this writer, the most likely scenario is that DG42 is an enzyme that synthesizes chitin oligomers in defined stages of the embryo. These could be functioning in a manner analogous to the signaling oligosaccharides of plants (39) and/or as a "primase" for hyaluronan synthesis. A less likely possibility is that the chitin synthesis is an *in vitro* artifact and that the DG42 gene product is a hyaluronan synthase. Time will tell.

Final resolution of these issues will require the synthesis of an epitope-tagged form of DG42 whose product could be directly isolated from vertebrate cells and tested for its activity. It would also be interesting to test if labeled chitooligosaccharides can actually prime hyaluronan synthesis in cell extracts. A more complete product characterization from the enzyme assays would also help. More direct studies of the nature of the core region of hyaluronan from a variety of sources would also be interesting.

Regardless of how all of this turns out, these polysaccharides will likely gain new respect as being more than just structural scaffolding, playing potentially important biological roles in vertebrate development. In this regard, it is worth pointing out our recent estimate that as much as 0.5–1% of the transcribed genome appears to be involved in the generation and recognition of the diversity of oligosaccharide synthesized by vertebrates (27). Indeed, many lines of evidence suggest that this investment of genomic material may have a significant impact on embryogenesis and morphogenesis (27).

Another interesting outcome of these studies is the suggestion that, like plants (39), animals may also generate "oligosaccharin" fragments that could potentially elicit biological

responses from target cells. The interested reader is also referred to the burgeoning literature on the diverse roles of hyaluronan-binding proteins in cell motility and adhesion, intercellular interactions, malignant transformation, and tumor metastasis (see refs. 25 and 40 for review and 41–53 for examples).

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