Hyaluronan (HA) is a large linear polymer of repeating disaccharides of glucuronic acid and GlcNAc. Although HA is widely distributed in vertebrate animals, it has not been found in invertebrates, including insect species. Insects utilize chitin, a repeating β-1,4-linked homopolymer of GlcNAc, as a major component of their exoskeleton. Recent studies illustrate the similarities in the biosynthetic mechanisms of HA and chitin and suggest that HA synthase (HAS) and chitin synthase have evolved from a common ancestral molecule. Although the biochemical properties and in vivo functions of HAS proteins have been extensively studied, the molecular basis for HA biosynthesis is not completely understood. For example, it is currently not clear if proper chain elongation and secretion of HA require other components in addition to HAS. Here, we demonstrate that a non-HA-synthesizing animal, the fruit fly Drosophila melanogaster, can produce HA in vivo when a single HAS protein is introduced. Expression of the mouse HAS2 gene in Drosophila tissues by the Gal4/UAS (upstream activating sequence) system resulted in massive HA accumulation in the extracellular space and caused various morphological defects. These morphological abnormalities were ascribed to disordered cell-cell communications due to accumulation of HA rather than disruption of heparan sulfate synthesis. We also show that adult wings with HA can hold a high level of water. These findings demonstrate that organisms synthesizing chitin (but not HA) are capable of producing HA that is structurally and functionally relevant to that in mammals. The ability of insect cells to produce HA supports the idea that in vivo HA biosynthesis does not require molecules other than the HAS protein. An alternative model is that Drosophila cells use endogenous components of the chitin biosynthetic machinery to produce and secrete HA.

Polysaccharides are used as major structural components of animal and plant bodies. In higher vertebrates, hyaluronan (HA), a high molecular mass glycosaminoglycan composed of repeating β-1,4-linked disaccharides of glucuronic acid (GlcUA) and β-1,3-linked GlcNAc, is found in the extracellular matrix in many tissues, including cartilage, synovial joint, and the vitreous of the eye (1). Despite wide distribution of HA in vertebrate animals, HA has not been found in invertebrates such as arthropod species. Instead of HA, crustaceans and arthropods produce chitin, a repeating β-1,4-linked homopolymer of GlcNAc, and deposit it in their exoskeleton. In addition, the plant cell wall is composed mainly of cellulose, β-1,4-glucan. These distinct types of polysaccharides share common important functions to determine and maintain the basic architecture of organisms, suggesting an evolutionarily conserved role of the polysaccharides as major structural constituents of multicellular organisms. Consistent with this idea, the biosynthetic mechanisms of these molecules show some intriguing similarities. All enzymes that synthesize HA, chitin, and cellulose are integral plasma membrane proteins and have the invariant amino acid residues QXXRW in their catalytic domains (2). Interestingly, one of the HA synthases (HASs), mouse HAS1, is indeed capable of synthesizing chito-oligosaccharides in vitro when only UDP-GlcNAc is supplied as substrate in the system (2). Based on these similarities, it has been proposed that three classes of glycosyltransferases (HASs, chitin synthases, and cellulose synthases) diverged from a common ancestral molecule.

HA is a multifunctional player in the vertebrate extracellular matrix. One of the important features of the HA network is its ability to hold a large amount of water, exhibiting viscoelastic properties. HA also directly affects cell behavior through its cell-surface receptors: CD44 (for review, see Ref. 3), RHAMM (receptor for HA-mediated motility) (4, 5), and Layilin (6). Recent studies on HASs, including molecular cloning of HAS cDNAs (7–13) and genes (14, 15) and functional analyses of each HAS gene (2, 16–20), illustrate the biological importance of HA. However, little is known about the mechanism of HA synthesis. Membrane extracts prepared from yeast cells expressing the Xenopus HAS (DG42) gene showed HA synthesizing activity in vitro solely in the presence of exogenously supplied substrates and magnesium ions (21). Furthermore, a purified single HAS1 protein or a HAS1 gene product of an in vitro transcription/translation system has been shown to have HA activity (2). These results indicate that the HAS protein alone can synthesize HA in vitro without any other protein factors. However, it is not clear whether other components are required for in vivo HA biosynthesis for proper chain elongation, termination, and secretion.

In this study, we demonstrate that a single mammalian HAS protein, HAS, has hyaluronan synthase activity, and a HAS1 gene product has hyaluronan synthase activity.
protein (HAS2) can efficiently synthesize and secrete HA in Drosophila, which is a chitin-synthesizing organism and which does not naturally produce HA. This implies that conversion of the biosynthetic machinery of chitin to that of HA can happen by a single molecule exchange: substitution of chitin synthase with HAS. HAS2 expression caused various morphological defects due to disruption of cell-cell communication. HA synthesized in fly tissues was able to retain a high level of water, which is the characteristic biophysical feature of this macromolecule. The ability of HAS2 to produce functional HA supports the idea that HAS is the only critical factor required for normal HA biosynthesis. Another interesting possibility is that HA is produced by HAS together with endogenous components involved in chitin biosynthesis.

EXPERIMENTAL PROCEDURES

Upstream Activating Sequence (UAS) Constructs and Ectopic Expression—The UAS-HAS2 transgene was constructed by cloning the full-length mouse HAS2 cDNA into the pUAST vector. Transgenic flies were obtained by P-element-mediated germ line transformation (22). In this study, we used two independent HAS2 transgenic strains (UAS-HAS2-1 and UAS-HAS2-2) that bear transgenic insertions at different chromosomal locations. The HAS2 gene was misexpressed in the Gal4/UAS system (23) using the following Gal4 drivers. 29BD-GALA is an enhancer trap line of P{GawB} and ubiquitously expresses a high level of Gal4 protein (24). engrailed-GAL4 and apterous-GAL4 were used to express HAS2 in the posterior and dorsal compartments of the wing disc, respectively. GMR-GAL4 and A0-GAL4 drive target gene expression in developing eyes and wings, respectively. To overexpress sugarless (sgl) gene, we used the transgenic strain bearing the sgl gene under the control of the ubiquitin promoter (ubi-sgl) (25).

Preparation and Quantification of Glycosaminoglycans—To quantify HA and chondroitin sulfate, crude glycosaminoglycans from 25 mg of lyophilized Drosophila larvae were prepared as described previously (26) and dissolved in 100 μl of H2O. A 20-μl portion of the crude glycosaminoglycans was digested with 2 turbitidity reducing unit of Streptomyces hyaluronidase in 100 μl of 50 mM acetate buffer (pH 6.0) at 60 °C for 1 h. The resulting hyaluronic acid tera- and hexasaccharides were then filtered with Ultrafree-MC (5000 molecular weight) filter. HA contained in the filtrates and the recovered glycosaminoglycans retained on the filter were digested separately with chondroitinases ABC and ACII and 5 μg of bovine serum albumin in 50 μl of 50 mM Tris-HCl (pH 8.0) at 37 °C for 2 h. The digests were filtered with Ultrafree-MC (5000 molecular weight limit) and unaltered disaccharides contained in the filtrates were determined by reverse-phase ion pair chromatography using a Senneghor-Pak Docol column equipped with a post-column fluorescence detector according to the method of Toyoda et al. (26) except for a slight modification of the elution conditions.

For preparation and quantification of heparan sulfate, a 10-μl portion of the crude glycosaminoglycans was digested with a mixture of 10 milligrams of heparitinase I, 5 milligrams of heparitinase II, and 10 milligrams of heparitinase III in 50 μl of 50 mM Tris-HCl (pH 7.2), 1 mM CaCl2, and 4 μg of bovine serum albumin at 37 °C for 16 h. HA was digested as described above.

Preparation and Quantification of Chitin—For the quantitative measurement of chitin, the amount of GlcNAc released by chitinase was measured by a colorimetric method. Lyophilized Drosophila larvae (25 mg, dry weight) were homogenized with 1.0 ml of acetone. The homogenate was washed with acetone and dried. The pellet was suspended in 1.0 ml of 0.1 N NaOH for 16 h at room temperature. After alkali treatment, 25 μl of 4 N acetic acid were added; actinase E was then added and incubated at 37 °C for 2 h. Insoluble material was isolated by centrifugation, washed twice with water, and resuspended in 400 μl of 200 mM acetate buffer (pH 5.0). Chitinase (2.0 mg) from Bacillus sp. dissolved in the same buffer (200 μM) was added, and the mixed solution was incubated at 37 °C for 90 min. After centrifugation, an 80-μl aliquot of each sample was assayed for GlcNAc by measuring the absorbance at 585 nm (27).

Gel Electrophoresis of HA—A 20-μl portion of the crude glycosaminoglycans prepared as described above was fractionated by 0.5% agarose gel electrophoresis (28). After electrophoresis, HA was blotted onto nylon membrane and detected by the ECL detection system with biotinylated HA-binding protein (b-HABP) (Seikagaku Co.) as a probe described previously (17). HA with average masses of 21.3, 14.1, 9.9, 6.4, 4.6, and 1.0 × 107 Da was used as a standard.

To test the sensitivity of detected bands to chitinase and hyaluronidase, the crude glycosaminoglycan fractions were treated with these enzymes before gel electrophoresis. The samples were incubated with 0.5 mg of chitinase from Bacillus sp. at 37 °C for 1 h. After incubation, the samples were incubated with 0.5% 3H2O2 in methanol. After washing with 0.3% Triton X-100 in phosphate-buffered saline, nonspecific binding was blocked with 10% goat serum in 0.3% Triton X-100 in phosphate-buffered saline. The discs were incubated with 2.5 μg/ml b-HABP in 10% goat serum overnight at 4 °C. After unbound probe was washed off, the discs were incubated with Avidin-Biotin Complex reagent (Vector Labs, Inc.) for 1 h, and b-HABP was detected by signal amplification with the apical surface of pupal retina as described previously (30).

Quantification of the H2O Content of Drosophila Adult Wings—Adult wings were collected from wild-type or 29BD-GALA/UAS-HAS2-2 males at 3–5 days after eclosion. To prevent evaporation of water from the tissue, wings were rapidly removed from the thorax at the base using forceps. Approximately 25–50 wings (0.1–0.2 mg) were used as a sample to measure the H2O content by the coulometric Karl-Fischer method (31, 32) using an A4-Q water content analyzer (Hiranuma Sangyo Co.). Immediately after the fresh weight of each sample was determined using an S4 Ultramicro balance (Sartorius Corp.), water was extracted from the wings and titrated with Hydranal Aqualyte RS (Merck, Darmstadt, Germany) as a titrant, which was measured by a water content analyzer (Kanto Kagaku Co.). The resulting water was used as a solvent. Sets of four and six samples were prepared for wild-type and 29BD-GALA/UAS-HAS2-2 wings, respectively, and measurements were performed independently.

RESULTS

HA Synthesis upon HAS2 Expression in Drosophila—A previous biochemical study on glycosaminoglycans from Drosophila showed that no detectable HA exists in this organism (26). We first asked whether Drosophila cells have the ability to synthesize HA when a HAS protein is introduced. We used the Gal4/UAS system (23) to drive expression of the mouse HAS2 gene in vivo. The HAS2 cDNA was ligated downstream of the UAS, and the resultant plasmid construct was integrated into the genomic DNA by P-element-mediated transformation. The established Drosophila strains bearing the UAS-HAS2 transgene were crossed with various Gal4 strains to express HAS genes ubiquitously or in a tissue-specific manner. To determine whether HA is synthesized in the HAS gene-expressing animals, we prepared crude glycosaminoglycans fractions from third instar larvae according to the method described previously (26) and quantified HA. A large amount of HA was detected in larvae obtained from crosses between the 29BD-GALA driver and two independent UAS-HAS2 transgenic strains, whereas no detectable HA was observed in the wild-type control animals (Table I).

We also examined HA synthesis in situ using b-HABP as a probe. HAS2 expression was induced by engrailed-GAL4, which drives Gal4 expression in the posterior compartment of imaginal discs (Fig. 1A). Histochemical staining showed specific binding of the b-HABP probe to the posterior half of the wing (Fig. 1B). This result confirmed that HA is synthesized in Drosophila tissues and accumulates at the sites of HAS2 expression. We also used this probe in a fluorescence detection system to determine the subcellular localization of HA. As shown in Fig. 1C, signals were detected mainly on the cell
Hyaluronan Production in Transgenic Drosophila

Crude glycosaminoglycans were prepared from wild-type and 29BD/HAS2 third instar larvae. The amounts of HA, chondroitin sulfate (CS), and heparan sulfate (HS) were determined by fluorescence disaccharide analysis. Chitin was also quantified as described under “Experimental Procedures.” Two independent transgenic strains for UAS-HAS2 (UAS-HAS2-1 and UAS-HAS2-2) were used for this analysis. ND, not detectable.

| Genotype         | HA (nmol/mg dry weight) | CS (nmol/mg dry weight) | HS (nmol/mg dry weight) | Chitin (pmol/mg dry weight) |
|------------------|-------------------------|-------------------------|-------------------------|-----------------------------|
| Wild-type        | ND                      | 0.54                    | 0.14                    | 5.8                         |
| 29BD/HAS2-1      | 0.20                    | 0.54                    | 0.10                    | 5.6                         |
| 29BD/HAS2-2      | 0.04                    | 0.56                    | 0.14                    | 5.4                         |

peaks with large molecular masses ranging from 1 × 10^5 to 2 × 10^6 Da (Fig. 1D, lane 2). The size distribution of HA prepared from 29BD-GAL4/UAS-HAS2 animals was smaller than that synthesized by the same enzyme in rat 3Y1 fibroblasts (molecular masses of >2 × 10^6 Da), but was comparable with that produced in vitro by membrane preparation of HAS2 transfectant (molecular masses of 2 × 10^5 to 2 × 10^6 Da) (17). On the other hand, a weak background signal was observed at a low molecular mass range in the wild-type control lane (Fig. 1D, lane 1). To determine whether this low molecular mass smear band reflects the existence of HA or other related molecules in the wild-type fraction, we tested its sensitivity to chitinase (Bacillus) and hyaluronidase (Streptomyces). As shown in lanes 3 and 5, the low molecular mass signals were not eliminated by incubation with chitinase or hyaluronidase, indicating that these signals do not represent either HA or chitin. In contrast, HA produced in HAS2-expressing animals was not affected by chitinase treatment (lane 4), but was completely degraded by adding hyaluronidase (lane 6). Although the nature of the low molecular mass band is unknown, it seems to be a background signal detectable only in blotting experiments because the b-HABP probe specifically recognized regions of the wing discs where HAS2 was expressed in situ (Fig. 1B) and did not stain wild-type discs (data not shown).

HAS2 Gene Expression Disrupts Morphogenesis in Drosophila Tissues—Overexpression of HAS2 using several different Gal4 drivers caused lethality and a variety of morphological defects in adult tissues (Fig. 2). Eye-specific expression of the HAS2 gene by GMR-GAL4 induced a so-called “rough eye” phenotype, characterized by reduced numbers of ommatidia and disordered ommatidial arrays of the compound eye (Fig. 2B). To further analyze this eye defect, we observed pupal retina stained with cobalt sulfide (Fig. 2, C and D). In contrast to wild-type retina, which showed precise and ordered patterns of differentiation of ommatidial components, HAS2-expressing eyes exhibited several distinct defects. These abnormalities included decreased numbers of cone cells, abnormal sizes and shapes of primary pigment cells, and ectopic interommatidial bristles. Overexpression of HAS2 in the developing wing using A9-GAL4 led to thickened and disarranged wing veins (Fig. 2, E and F). When HAS2 expression was induced in the dorsal part of wing imaginal discs by apterous-GAL4, the wings failed to extend. In addition, the patterns and formation of notal bristles were disrupted; most large mechanosensory bristles (macrochaetae) were lost or shortened with abruptly ended tips (Fig. 2, G and H). In this study, we used two independent HAS2 transgenic strains, UAS-HAS2-1 and UAS-HAS2-2. The phenotypes associated with HAS2 expression by these two transgenes were fundamentally similar, but different in severity (Table II). Expression of UAS-HAS2-1, which produces higher levels of HA, resulted in more severe expressivity and higher penetrance compared with that of UAS-HAS2-2.

All these abnormalities are known to be caused by defective intercellular signaling. For example, during eye development, differentiation of cone and pigment cells requires activation of the Drosophila epidermal growth factor receptor (DER), and

![Figure 1. HA synthesis upon mouse HAS2 expression in Drosophila wing discs.](http://www.jbc.org/)

A) The posterior compartment-specific pattern of engrailed-GAL4-driven expression of HAS2 in the wing disc. The expression pattern of lacZ in engrailed-GAL4/UAS-lacZ wing discs was monitored by β-galactosidase activity staining. B) HA localization in the engrailed-GAL4/UAS-HAS2-2 wing disc determined by staining using b-HABP as a probe. HA was specifically detected in the posterior region where HAS2 was overexpressed by engrailed-GAL4. C, subcellular localization of HA in the HAS2-expressing disc. The fluorescent signal of HA was detected on the cell surface and/or in the extracellular space. D, agarose gel electrophoresis of HA synthesized in Drosophila. Crude glycosaminoglycan fractions were prepared from wild-type (lanes 1, 3, and 5) and 29BD-GAL4/UAS-HAS2-2 (lanes 2, 4, and 6) animals as described under “Experimental Procedures” and separated by agarose gel electrophoresis. Samples loaded in lanes 3 and 4 were treated with chitinase (from Bacillus sp.) before gel electrophoresis; those loaded in lanes 5 and 6 were digested with both chitinase and Streptomyces hyaluronidase (HAs). HA was blotted onto nylon membrane and detected using the RCL detection system with b-HABP as a probe. HA with average masses of 21.3, 14.1, 9.9, 6.4, 4.6, and 1.0 × 10^5 Da was used as a standard.
sulfate, a different class of glycosaminoglycans, requires the common substrates UDP-GlcUA and UDP-GlcNAc, a possible explanation for these phenotypes could be interference with heparan sulfate synthesis; high levels of HAS protein may compete for the substrates with the endogenous heparan sulfate biosynthetic machinery. Indeed, a number of studies have demonstrated that heparan sulfate plays a critical role in Drosophila morphogenesis (for reviews, see Refs. 38–41). Similarly, synthesis of chondroitin sulfate and chitin, which need UDP-GlcUA and UDP-GlcNAc, respectively, may also be affected. To determine the effects of HAS2 expression on the biosynthesis of chondroitin sulfate, heparan sulfate, and chitin, we measured the levels of these polysaccharides in the HAS2-expressing animals. As shown in Table I, we did not detect a significant change in the levels of these molecules upon expression of HAS2, although UAS-HAS2-1/+;29BD-GAL4/+ showed a moderately reduced level of heparan sulfate.

Overexpression of UDP-glucose Dehydrogenase Enhances the Phenotype of HAS2-expressing Animals—Several lines of information suggest that the moderate reduction of heparan sulfate in UAS-HAS2-1/+;29BD-GAL4/+ animals is unlikely to cause the morphological defects in the HAS2-expressing animals. First, reduced levels of heparan sulfate cannot explain the various phenotypes of 29BD-GAL4/UAS-HAS2-2, which showed a normal level of heparan sulfate (Table I). Second, we have several other transgenic strains that produce higher levels of HA and also show wild-type levels of heparan sulfate (data not shown); thus, HA production and heparan sulfate reduction do not seem to be directly correlated. To further examine whether the phenotypes associated with HAS2 expression depend on the levels of HA or heparan sulfate, we performed a sensitive genetic assay in which the cellular levels of UDP-GlcUA were manipulated. If the HAS2 overexpression phenotypes are caused by HA accumulation on the cell surface, these phenotypes should be enhanced by increasing the UDP-GlcUA level and should be suppressed by its reduction. The reverse will happen if these defects are consequences of loss of substrates and defective heparan sulfate-dependent signaling.

Based on these criteria, we genetically manipulated the dosage of sgl, which encodes a UDP-glucose dehydrogenase, an essential enzyme for UDP-GlcUA biosynthesis (25, 42–45), and examined its effect on the rough eye phenotype of GMR-GAL4; UAS-HAS2/+; UAS-HAS2/+ animals. As depicted in Fig. 3B, we did not detect a significant change in the phenotype by deleting one copy of sgl. On the other hand, overexpression of sgl by the transgene under the control of the ubiquitin promoter (ubi-sgl) (25) dramatically enhanced the eye defects, resulting in gross defects in eye formation with necrosis (Fig. 3D). This observation indicates that HA accumulation (but not disrupted heparan sulfate synthesis) is responsible for the morphological abnormalities in the HAS2-expressing animals.

HA-synthesizing Wings Accumulate High Levels of Water—

The ability of HA to hold a large amount of water is an important biophysical property as a structural constituent of the extracellular matrix. We noticed that HAS2 overexpression by the UAS-HAS2-2 transgene under the control of 29BD-GAL4 at 20 °C induced the characteristic wing phenotypes (Fig. 4, B and B’). These wings were thick and partly wrinkled, and the dorsal and ventral epithelial sheets were detached from each other. In addition, they were opaque compared with wild-type wings, which were transparent. Overall, the wings appeared to be swollen with fluid in their interior. Wings from engrailed-GAL4/+;UAS-HAS2-2/+ adults also exhibited a similar phenotype only in the posterior compartment (data not shown), confirming that this phenotype is associated with HA accumulation. To determine whether these wings show in-
creased water content, we collected wild-type and 29BD-GAL4/UAS-HAS2-2 wings and measured H2O in these tissues by the Karl-Fischer method (31, 32). Each error bar represents the S.E. of four and six measurements of wild-type and UAS-HAS2-2 wings, respectively. The difference among them is significant at the 1% level as determined by Mann-Whitney’s U test (p = 0.0089).

DISCUSSION

Insects produce cuticle, the chitin-based exoskeleton that prevents desiccation of body moisture. This structure enables these species to attain light body weight and to accommodate a large variety of living space. On the other hand, higher vertebrate animals employ HA, which ensures water inside the body. Thus, the life styles of animals are not unrelated to the polysaccharides they produce, and diversion of HA- and chitin-producing organisms could be one of the key steps in animal evolution. Recent studies suggest that the HAS and chitin synthase genes have evolved from a common origin. Here, we have provided evidence that HA can be produced by introducing a single HAS gene into a chitin-synthesizing multicellular organism, the Drosophila fruit fly, which normally does not synthesize HA. The HAS2 enzyme was active in such a heterologous system and efficiently synthesized a high level of HA without exogenously supplied substrates or primers. The observation that HA products were deposited on the cell surface implies that HAS acts in Drosophila cells as it functions in mammalian cells: it polymerizes HA chains at the inner face of the plasma membrane and coordinately secretes HA out of the cell. This result is significant because it is currently unclear whether proper elongation and secretion of HA require other proteinaceous and non-proteinaceous components in addition to HAS. Our finding of efficient HA production and secretion in insect cells strongly supports that such additional factors are not essential for normal HA biosynthesis. However, we cannot exclude the possibility that HAS2 accomplishes HA synthesis by utilizing the chitin biosynthetic machinery in Drosophila cells. Additional studies on the biosynthetic machineries of HA and chitin will clarify this point. Thus, the Drosophila system will provide new insights into unsolved problems regarding HA biosynthesis.

The HAS2-overexpressing flies showed a variety of phenotypes in many tissues. The biochemical and genetic experiments showed that these phenotypes are caused by accumulation of HA in tissues, but not by lack of chondroitin sulfate, heparan sulfate, or chitin. How did HA synthesis induce such phenotypes? In the HA network in a physiological solution, diffusion rates of macromolecules such as proteins are expected to be slow, and the concentration of these molecules will be lower in the network compared with an HA-free environment. This would explain the observed morphological defects. The abnormalities caused by HAS2 expression are reminiscent of phenotypes caused by disruption of intercellular signaling mediated by extracellular or cell-surface signaling molecules, the movement of which will be restricted in the HA network. For
The eye phenotype of HAS2-overexpressing animals was strongly enhanced by coexpression of the sgl gene, which encodes a UDP-glucose dehydrogenase. It is worth noting that a substantial effect of increasing the level of UDP-glucose dehydrogenase on the phenotype is consistent with the prediction that substrate availability is a limiting factor in HA biosynthesis (44). Although we have not tested the effects of alterations in UDP-GlcNAc levels on HA deposition, it is possible that the cytosolic concentration of UDP-GlcNAc might be high in insect cells that synthesize chitin, a GlcNAc polymer, at the inner face of the plasma membrane and is therefore less critical than the UDP-GlcUA level.

The consequences of lack or elevation of HA synthesis have become the recent focus of intense research interest. Cameni

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In Vivo Hyaluronan Synthesis upon Expression of the Mammalian Hyaluronan Synthase Gene in Drosophila
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