Hyaluronan Synthase 1 (HAS1) Requires Higher Cellular UDP-GlcNAc Concentration than HAS2 and HAS3*[S]

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Background: HAS isoenzymes differ in enzymatic activity and regulation.
Results: HAS1 requires higher UDP-sugar concentration than HAS2 and HAS3.
Conclusion: HAS1 activity is highly dependent, and its expression correlates with cellular UDP-sugar supply.
Significance: Enhanced UDP-sugar levels are potential mediators of enhanced hyaluronan secretion in cancer and inflammation.

Mammals have three homologous genes encoding proteins with hyaluronan synthase activity (*Has1–3*), all producing an identical polymer from UDP-*N*-acetylglucosamine and UDP-glucuronic acid. To compare the properties of these isoenzymes, COS-1 cells, with minor endogenous hyaluronan synthesis, were transfected with human *Has1–3* isoenzymes. HAS1 was almost unable to secrete hyaluronan or form a hyaluronan coat, in contrast to HAS2 and HAS3. This failure of HAS1 to synthesize hyaluronan was compensated by increasing the cellular content of UDP-*N*-acetyl glucosamine by ~10-fold with 1 mM glucosamine in the growth medium. Hyaluronan synthesis driven by HAS2 was less affected by glucosamine addition, and HAS3 was not affected at all. Glucose-free medium, leading to depletion of the UDP-sugars, markedly reduced hyaluronan synthesis by all HAS isoenzymes while raising its concentration from 5 to 25 mM had a moderate stimulatory effect. The results indicate that HAS1 is almost inactive in cells with low UDP-sugar supply, HAS2 activity increases with UDP-sugars, and HAS3 produces hyaluronan at high speed even with minimum substrate content. Transfected *Has2* and particularly *Has3* consumed enough UDP-sugars to reduce their content in COS-1 cells. Comparison of different human cell types revealed ~50-fold differences in the content of UDP-*N*-acetylhexosamines and UDP-glucuronic acid, correlating with the expression level of *Has1*, suggesting cellular coordination between *Has1* expression and the content of UDP-sugars.

Hyaluronan is an extremely long, linear carbohydrate polymer abundant in the extracellular space of vertebrate tissues. High levels of this glycosaminoglycan exist in synovial and mesothelial fluids (1), in resilient tissues like dermis (2) and cartilage (3), and in the transparent vitreous body of the eye

where it was originally discovered (4). Because of its physico-chemical properties, hyaluronan can influence many cellular functions including migration, proliferation, and adhesion, but it may also act as a signaling molecule. Hyaluronan is produced by three isoforms of hyaluronan synthase (HAS1, 2, and 3),[3] these enzymes act on the inner face of plasma membrane (5) and simultaneously translocate the growing polysaccharide through plasma membrane into extracellular space (6, 7).

The three members of the *Has* gene family are located in different chromosomes, expressed at specific stages of organ development (8) and are subject to regulation by different hormonal and environmental signals (9). Although all three HAS isoforms resemble each other in protein sequence and function, some differences have been noted in enzyme stability, chain elongation rate, length of the chain, and substrate-dependent enzyme properties (10–13). Their subcellular locations are also slightly different (14). However, the biological importance of having the three different isoforms is not completely clear.

A pericellular hyaluronan coat is produced as a result of HAS activity on plasma membrane. A large coat, visualized by exclusion of particles allowed to sediment on the cultures (15), is also induced when cells are transfected with *Has2* or *Has3* (12, 16, 17). In contrast, the coat created by transfection of *Has1* is small (12, 16) or completely missing (11). The minor coat corresponds to generally lower hyaluronan synthesis rate of HAS1 (12, 16).

The two glycosyltransferase activities in HAS add glucuronic acid and *N*-acetylglucosamine into their alternating positions in the chain, using UDP-glucuronic acid (UDP-GlcUA) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) as substrates (18). Sufficient supply of both UDP-GlcUA and UDP-GlcNAc is important for hyaluronan synthesis. Indeed, two relatively specific inhibitors of hyaluronan synthesis target these UDP-sugars; 4-methylumbelliferone depletes UDP-GlcUA (19–21), and mannose reduces the cellular content of UDP-GlcNAc (22). The specific sensitivity of hyaluronan synthesis to the inhibitors

[3] The abbreviations used are: HAS, hyaluronan synthase; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; UDP-GlcUA, uridine 5′-diphosphoglucoronic acid; UDP-HexNAc, UDP-*N*-acetylhexosamines; MEM, minimum essential medium; HABC, hyaluronan-binding complex; ANOVA, analysis of variance.

likely comes from the fact that HAS works on the cytosolic pool of the UDP-sugars, whereas most other glycosylations take place in Golgi apparatus, a privileged compartment in this respect because of the high affinity pumps that import nucleotide sugars from cytosol (23). The importance of the UDP-sugars for HAS activity is also reflected in the interesting finding that depletion of UDP-GlcUA (14) and UDP-GlcNAc[4] prevents the access or stay of HAS in plasma membrane.

The cellular content of UDP-GlcNAc is highest among the different nucleotide sugars. However, the HAS K_m for UDP-GlcNAc is also relatively high, probably necessitating the abundance of this substrate for efficient hyaluronan synthesis (16). UDP-GlcUA is usually present in quantities 10–30% of UDP-GlcNAc, but its K_m value is correspondingly lower, so that both substrates are expected to be in balanced supply for hyaluronan synthesis. Although the K_m values for UDP-GlcUA are close to each other within the HAS family, interesting differences between members of the family were reported in the K_m for UDP-GlcNAc. HAS1 showed a K_m value for UDP-GlcNAc up to 10-fold higher than HAS3, whereas the value of HAS2 was slightly higher than that of HAS3 (16).

These *in vitro* observations suggested that HAS1 activity requires a relatively high concentration for UDP-GlcNAc. As far as we know, the possible biological implications of this finding have not been explored in live cells. When our transfections of *Has1–3* in identical plasmid and cellular backgrounds consistently resulted in little if any hyaluronan synthesis with HAS1, we raised the hypothesis that it was due to a relative shortage of UDP-GlcNAc. Manipulation of the cellular UDP-GlcNAc pool with glucosamine addition and glucose deprivation confirmed the different UDP-sugar requirements of the HAS isoenzymes *in vivo*. Moreover, it turned out that hyaluronan synthesis can consume a large proportion of cellular UDP-sugars and that significant levels of HAS1 were not expressed in cell types with UDP-GlcNAc concentrations probably insufficient for its hyaluronan synthesis, suggesting coordinated regulation of *Has1* expression and the cellular pool size of UDP-GlcNAc.

MATERIALS AND METHODS

Cell Culture—COS-1 cells were cultured in DMEM (high glucose; Invitrogen) supplemented with 10% FBS (HyClone; Thermo Fisher Scientific, Waltham, MA), 2 mM glutamine, 50 μg/ml streptomycin sulfate, and 50 units/ml penicillin (EuroClone, Milan, Italy). The cells were passaged twice a week at a 1:5 split ratio using 0.05% trypsin (w/v) 0.02% EDTA (w/v) (Biochrom AG, Berlin, Germany). For glucose concentration experiments, a glucose-free culture medium (Invitrogen) was used. Human mesothelial cells (LP-9) were cultured in MCDB110 and 199 media (1:1) (Sigma), supplemented with 15% FBS, 2 mM glutamine, 50 μg/ml streptomycin sulfate, 50 units/ml penicillin, 10 ng/ml epidermal growth factor (Sigma), and 0.05 μg/ml hydrocorticone (Sigma). Human breast adenocarcinoma cells (MCF-7) were cultured in minimum essential medium (MEMα; Invitrogen) supplemented with 5% FBS, 2 mM gluta-

mine, 50 μg/ml streptomycin sulfate, and 50 μg/ml penicillin. Human cutaneous normal and tumor stromal fibroblasts (from Dr. Reidar Grenman, Turku, Finland) were cultured in DMEM (high glucose; Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 50 μg/ml streptomycin sulfate, 50 units/ml penicillin, and 1% nonessential amino acids (Invitrogen). Human diploid lung fibroblasts (WI-38) were cultured in Earle's MEM (Invitrogen) supplemented with 20% FBS, 2 mM glutamine, 50 μg/ml streptomycin sulfate, 50 units/ml penicillin, 1% nonessential amino acids, and 1% MEM vitamins (Invitrogen). Human chondrosarcoma cells were cultured in MEMα (modification with glutamine) (Hyclone) supplemented with 10% FBS, 50 μg/ml streptomycin sulfate, and 50 units/ml penicillin. Human keratinocytes (HaCaT cells), developed by Boukamp *et al.* (52) were obtained from Cell Line Service (Heidelberg, Germany) and cultured in DMEM (high glucose; Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 50 μg/ml streptomycin sulfate, and 50 units/ml penicillin. When different cell types were compared with each other, the cells were grown to 80–90% confluency before sample preparation.

Dendra2 Constructs—The functional ORFs of full-length human hyaluronan synthase genes (*Has1*, NCBI nucleotide accession number NM_001523), *Has2* (NM_005328), and *Has3* (NM_005329) were amplified from human cDNA and ligated in frame with the pDendra2-C vector (Evrogen, Moscow, Russia) to form fusion genes of Dendra2 and *Has1–3*. The integrity of the constructs were initially checked by restriction endonuclease digestion analysis and finally analyzed by sequencing the whole ORF insert.

Transfections—COS-1 cells were seeded on eight-chamber slides (Ibidi GmbH, Martinsried, Germany) for confocal microscopy or on 24-well plates (CELL STAR®; Greiner Bio-One, Kremsmunster, Austria) for biochemical analyses. The next day, the cells were transfected with Exgen transfection reagent (Fermentas, St. Leon-Rot, Germany). A day after transfection, the cells were utilized for experiments.

Visualization of Hyaluronan in Live Cells with Fluorescent HABC—For visualization of hyaluronan on live cells, a fluorescent group (Alexa Fluor® 568) was directly coupled to HABC, as described earlier (24). Live cell cultures grown on chambered coverglasses (Ibidi GmbH) were incubated for 2 h in 37 °C with 5 μg/ml of fluorescent HABC in culture medium. Before live cell confocal imaging, DRAQ5™ DNA label (2.5 μM; Biostatus Ltd., Leicesterchire, UK) was added for labeling of nuclei.

Immunostaining of Endogenous HASs—The cells grown on chambered coverglasses (Ibidi) were fixed with 4% paraformaldehyde, permeabilized for 20 min at room temperature with 0.1% Triton-X-100, blocked with 1% BSA, and incubated overnight at 4 °C with a polyclonal antibodies for hyaluronan synthase HAS1 (2 μg/ml, sc-34021; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), HAS2 (2 μg/ml, sc-34067; Santa Cruz), or HAS3 (2 μg/ml sc-34204; Santa Cruz), diluted in 1% BSA. After washing, the cells were incubated for 1 h with secondary antibodies (Texas Red anti-goat; Vector Laboratories Inc., Burlingame, CA).

Confocal Microscopy and Image Analysis—The fluorescent images were obtained with 40× NA 1.3 oil objective on a Zeiss Axio Observer inverted microscope equipped with a Zeiss LSM

700 confocal module. For live cell imaging, a Zeiss XL-LSM S1 incubator with temperature and CO_2 control was utilized. ZEN 2009 software (Zeiss) was used for image processing and measurements. Total intensity (mean intensity $\times$ area) of Dendra2 fluorescence signal at 488 nm and hyaluronan coat (Alexa Fluor® 568) was quantified from each image of 320- $\times$ 320-μm areas, and the total fluorescence intensity was divided by the total number of positive cells. Transfection efficiency was evaluated by dividing the number of Dendra2-positive cells with the total cell number, visualized with DRAQ5 nuclear staining as described above.

Hyaluronan ELISA—For measurement of hyaluronan secretion into culture media, 80,000 COS-1 cells were plated on each well of 24-well plates (CELL STAR®; Greiner Bio-One) and transfected the next day with Dendra2-*Has* constructs. A day after transfection, a fresh culture medium with different concentrations of glucosamine (Sigma) or of glucose (Sigma) was changed. After 6 h of incubation, the culture media were collected and assayed for the concentration of hyaluronan using a sandwich-type ELISA as described previously (25).

In Vitro HAS Activity in Isolated Membrane Preparations— Hyaluronan synthesis in COS-1 cell membrane preparations was performed as described before with minor changes (10). The cells were plated in 10-cm cell culture dishes and transfected with Dendra2-HAS1 or Dendra2-HAS3 plasmids as described above. After an overnight incubation, the cells were washed with PBS and disrupted by sonication in a buffer containing 10 mM HEPES, pH 7.1, with 0.5 mM DTT, 0.25 M sucrose, and a proteinase inhibitor mixture (Sigma). A crude membrane fraction was obtained by ultracentrifugation of the suspension 137,000 $\times$ *g* for 1 h at 4 °C. The pellet was resuspended into 40 mM HEPES, pH 7.1, 8 mM DTT, 23 mM MgCl, and the proteinase inhibitor mixture. Final concentrations of 1 mM UDP-GlcUA and 0, 0.5, or 1.0 mM of UDP-GlcNAc (CarboSource Services, University of Georgia, supported in part by NSF-RCN Grant 0090281) were obtained from stocks dissolved in H_2O. Following 1 h of incubation at 37 °C, 6 volumes of 100% ethanol was added, kept overnight at -20 °C, and centrifuged 16,000 $\times$ *g* for 30 min. The pellet was air-dried and suspended in PBS. An aliquot was taken to assay hyaluronan content and another to measure Dendra2 fluorescence with a Victor3 multilabel plate reader (PerkinElmer Life Sciences). The hyaluronan content produced during the 1-h incubation was normalized to the Dendra2 fluorescence of the sample.

UDP-Sugars—When different cell lines were compared, cells were seeded on 10-cm plates and cultured until 90% confluence, counted from one plate, whereas cells from parallel plates were used to isolate and measure UDP-sugars as described earlier (26), using the sodium hydroxide/sodium acetate gradient as originally described by Tomiya *et al.* (27) with some modifications. Because with this method the isomers UDP-GalNAc and UDP-GlcNAc were incompletely separated in a part of the samples, the results are presented as UDP-HexNAc, which includes both. These isomers exist in equilibrium strongly dominated by UDP-GlcNAc in all cell types.

In other experiments, cells were grown on 6-cm plates until 80–90% confluence. After 6-h treatments, one plate was used for cell counting and others for UDP-sugar isolation. Cultures

TABLE 1
PCR primer sequences

Gene	Primer sequences (5′ to 3′)
Has1	
Forward	CAAGATTCTTCAGTCTGGAC
Reverse	TAAGAACGAGGAGAAAGCAG
Has2	
Forward	CAGAATCCAAACAGACAGTTC
Reverse	TAAGGTGTTGTGTGTGACTGA
Has3	
Forward	CTTAAGGGTTGCTTGCTTGC
Reverse	GTTCGTGGGAGATGAAGGAA
RPLPO	
Forward	GTGGTGATACCTAAAGCCTG
Reverse	AGATGCAGCAGATCCGCAT

were washed with cold PBS on ice, and 80 μl of 0.1 mM xylose or 40 μl of arabinose (CarboSource Services) was added as an internal standard. Cold acetonitrile (2 ml) was added to precipitate proteins and extract the UDP-sugars. The cells were scraped off, and the plate was further washed with 1 ml of deionized water. The suspension was centrifuged (6000 $\times$ *g* for 20 min), and the supernatant was transferred to a clean tube, evaporated in a vacuum centrifuge, and dissolved in PBS. Alternatively, samples for the glucose concentration experiments were scraped directly into PBS and sonicated before centrifugation (6000 $\times$ *g* for 20 min). The samples were further purified with Superclean Envi-Carb SPE-tubes (Sigma) as described previously (28). Eluted samples were evaporated by vacuum centrifugation and dissolved in water for anion exchange HPLC with a CarpoPac™ PA1 column (4 $\times$ 250 mm; Dionex, Sunnyvale, CA). A program (with improved separation between UDP-GlcNAc and UDP-GalNAc) had a gradient made of ultrapure H_2O (A), 1.3 M sodium borate, pH 7.0 (B), and 1 M sodium acetate, pH 7.0 (C). The column was equilibrated with 25%:75%:0% (v/v/v) of buffers A, B, and C, and elution was performed with the following program: T0 = 30%:70%:0%, T1 = 25%:75%:0%, T11 = 25%:75%:0%, T28 = 21%:34%:45%, T40 = 12%:20%:68%, T45 = 10%:10%:80%, T50 = 5%:5%:90%, and T55 = 30%:70%:0%. Integrated peak areas of the UDP-sugars were compared with that of the internal standard.

RNA Extraction and PCR—Total RNA was extracted with Eurozol (Euroclone), and the amplifications were performed in an MX3000P thermal cycler (Stratagene, La Jolla, CA), using the Absolute™Max two-step quantitative RT-PCR SYBR® Green kit (Abgene, Epsom Surrey, UK). Ribosomal protein, large, PO (*RPLPO*) gene was used as a control, and the results are shown as percentages of target gene expression of *RPLPO* expression level. PCR product quality was monitored using post-PCR melt curve analysis. Gene specific primers are shown in Table 1.

Statistics—The significance of differences between the groups was tested using one-way analysis of variance (ANOVA) with Dunnett's post hoc test or two-way analysis of variance with Bonferroni post hoc test. Statistical tests were performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Pearson correlations and their significance were performed in SPSS (v.17.0; SPSS Inc., Chicago, IL).
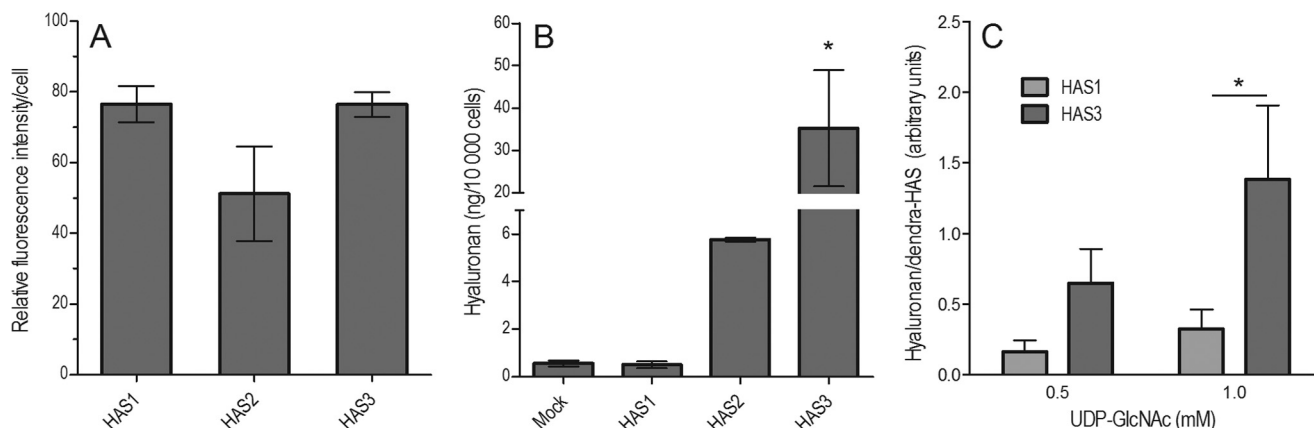
FIGURE 1. **Expression of Dendra-HAS1–3.** *A*, average integrated fluorescence per COS-1 cell was determined by microscopy 24 h after transfection of the different *Has* isoenzymes as Dendra2 fusion plasmids. The means ± S.E. of three experiments are shown. *B*, hyaluronan synthesis into growth medium was measured during a 6-h incubation period on the day after transfection of *Has1–3* and empty (Mock) plasmids transfected into COS-1 cells. The means ± S.E. of three independent experiments, each with two to three replicates, are presented. Statistical significance was measured with ANOVA and Dunnett's post hoc test. *, $p < 0.05$ as compared with mock transfection. *C*, *in vitro* activities of HAS1 and HAS3 in isolated membrane preparations were determined with ELISA after 1 h of incubation with 1 mM UDP-GlcUA and the indicated concentrations of UDP-GlcNAc. The values are normalized to controls (0 mM UDP-GlcNAc) and Dendra2 fluorescence of the samples. The means ± S.E. of four independent experiments are shown. Statistical significance was measured with two-way ANOVA and Bonferroni post hoc test. *, $p < 0.05$ as HAS1 compared with HAS3.
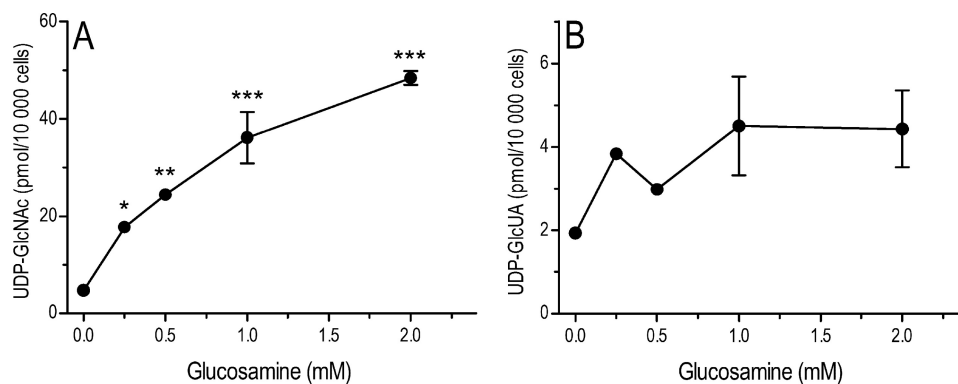

FIGURE 2. **Effect of glucosamine on UDP-sugars in COS-1 cells.** The levels of cellular UDP-GlcNAc (*A*) and UDP-GlcUA (*B*) were measured with HPLC after a 6-h treatment with 0 to 2 mM glucosamine in growth medium containing 25 mM glucose. The means ± S.E. of two independent experiments are presented, each with two replicates. Overall, glucosamine significantly influenced UDP-GlcNAc levels ($p = 0.0004$) measured by ANOVA. The increase in UDP-GlcUA levels was not significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as compared with 0 mM glucosamine by Dunnett's post hoc test.

RESULTS

Transfection of Has1–3 in COS-1 Cells—To compare the individual HAS enzymes for their properties concerning the synthesis of hyaluronan, we put human *Has1–3* genes in the same plasmid background (Dendra2). To avoid interference by endogenous HASs, the constructs were transfected into COS-1 cells, which synthesize very little hyaluronan. The levels of the Dendra2-HAS1, -HAS2, and -HAS3 proteins in cells transfected with identical amounts of the plasmid were not markedly different, as indicated by the integrated fluorescence intensities of the positive cells (Fig. 1*A*). Transfection efficiencies of the Dendra2-HAS1, Dendra2-HAS2, and Dendra-HAS3 plasmids were also similar: 31 ± 9, 16 ± 5, and 28 ± 5%, respectively.

Hyaluronan secreted into the growth medium during a 6-h incubation period, started 24 h after transfection, was measured using the ELISA described under "Materials and Methods." The secretion was very low in mock transfected (empty plasmid) cultures (Fig. 1*B*). Hyaluronan synthesis remained very small in *Has1*-transfected cells, in contrast to those transfected with *Has2* and especially those receiving *Has3*, with the latter showing a relatively high synthesis rate.

The weaker synthesis of hyaluronan by HAS1 was not due to lower expression level of the HAS protein, as evident in Fig. 1*A*. It was also not specific for this plasmid background because the same was observed by transfections with untagged *Has1* (data not shown). Furthermore, this property was not limited to COS-1 cells, because transfection of Dendra2-*Has1* and untagged *Has1* into MCF-7 cells resulted in very little additional hyaluronan production, as compared with *Has2* and *Has3* (supplemental Fig. S1). The distribution of endogenous HASs was similar to that of the Dendra2-HASs, both prominent in intracellular compartment, like Golgi complex, indicating that the tag had no obvious impact on intracellular trafficking (supplemental Fig. S3).

To confirm the lower activity of HAS1 protein *in vitro*, membranes were isolated from COS-1 cells transfected with Dendra2-HAS1 and -HAS3 and analyzed for HAS activity *in vitro*. Incubation of Dendra2-HAS1 proteins with unlimiting UDP-GlcUA concentration and increasing concentrations of UDP-GlcNAc produced significantly less hyaluronan than HAS3 in identical conditions (Fig. 1*C*). These results are in line with those obtained earlier using mouse *Has* genes (16), dem-
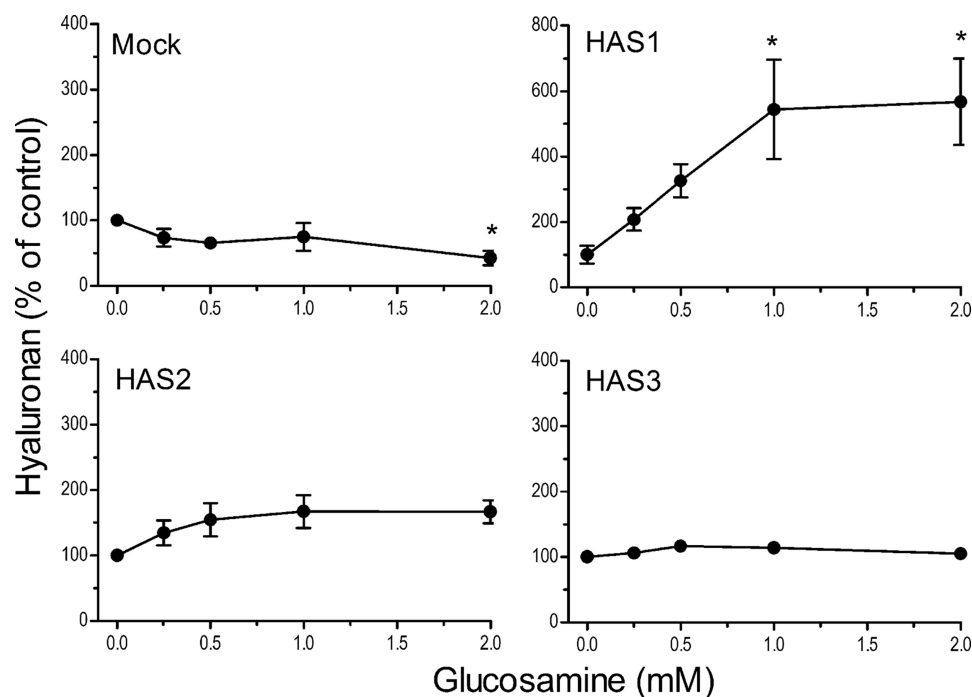
FIGURE 3. **Effect of glucosamine on hyaluronan synthesis by HAS1-, HAS2-, and HAS3-transfected COS-1 cells.** The day following transfection, normal growth medium (25 mM glucose) was supplied with 0–2 mM glucosamine and incubated for 6 h before the assay of secreted hyaluronan. The means ± S.E. of three (HAS2) or four (mock, HAS1, and HAS3) independent experiments, each with two replicates, are presented. A significant change in hyaluronan production was found in *Has1*-transfected cells ($p = 0.012$, measured by ANOVA). *, $p < 0.05$ compared with 0 mM glucosamine by Dunnett's post hoc test.

onstrating that both human and mouse enzymes have similar substrate demands and that the Dendra2 tag does not influence the HAS protein function. These results indicate that the lower synthesis rate and higher UDP-GlcNAc requirement is due to the intrinsic properties of the HAS1 protein.

Substrate Sensitivity of HAS1–3—The rate of hyaluronan synthesis depends on the supply of the substrates UDP-GlcUA and UDP-GlcNAc (29, 30). This and the fact that the three HAS enzymes show different enzyme activities also when analyzed on membrane preparations *in vitro* (16) led us to seek explanations from cellular UDP-sugar supply for the differences between HAS1–3 isoenzymes.

The contents of UDP-GlcNAc and UDP-GlcUA in COS-1 cells, ~5 and 2 pmol/10,000 cells, respectively (Fig. 2), were relatively low as compared, for example, with epidermal keratinocytes containing two to three times higher levels (29). Cellular UDP-GlcNAc can be increased by adding glucosamine, which bypasses the normal regulation of hexosamine synthesis at its rate-limiting enzyme glutamine fructose-6-phosphate amidotransferase (29). The addition of glucosamine in the growth medium also augments the cellular pool of UDP-GlcUA (29), thus increasing both substrates for hyaluronan synthesis. Fig. 2 shows how the contents of UDP-GlcNAc and UDP-GlcUA in COS-1 cells respond to 6-h treatments of different glucosamine concentrations. An approximately 4-fold increase in UDP-GlcNAc was detected already at 0.25 mM glucosamine, and more than 10-fold at 2 mM glucosamine (Fig. 2A). The increase of UDP-GlcUA was not significant, although ~2-fold elevation at 1–2 mM glucosamine concentrations was detected (Fig. 2B).

Changes in hyaluronan synthesis induced by HAS1, HAS2, and HAS3 in the different substrate concentrations were stud-

ied during a 6-h period following introduction of glucosamine. The elevated UDP-sugar levels caused a dose-dependent, up to 4-fold increase in hyaluronan synthesis by HAS1, whereas the increase by HAS2 was 1.6-fold, and virtually no change in the rate of hyaluronan production was found by HAS3 (Fig. 3). This suggests that HAS1 has a markedly higher requirement for the UDP-sugar concentration as compared with HAS2 and HAS3.

Curiously, the addition of glucosamine further decreased the originally minimal hyaluronan production in the mock transfected cultures (Fig. 3). Because it was recently shown that UDP-GlcNAc acts as a transcriptional feedback regulator of *Has2* expression (34), we analyzed the expression of the endogenous *Has* genes in COS cells using human primers. Indeed, glucosamine markedly inhibited the expression of *Has2*, probably contributing to the unexpected decline in hyaluronan production by mock transfected cells (supplemental Fig. S2).

Hyaluronan Coats Produced by HAS1–3—During its synthesis at cell surface, hyaluronan is attached to HAS and can be detected on live cells by a fluorescently labeled HABC (24). Microscopic examination confirmed that cells transfected with HAS2 and HAS3 (*green*) show a prominent hyaluronan coat (*red*), indicating ongoing hyaluronan synthesis (Fig. 4A). The coat present on *Has2*-transfected cells appeared larger and more diffuse than that in *Has3*-transfected cells. Integrated total fluorescence of hyaluronan per cell showed that the coats on HAS2-positive cells were indeed somewhat larger than the coats produced by HAS3 (Fig. 4B). In the standard culture conditions, the size of the HAS2- and HAS3-induced coats did not depend on cellular UDP-sugar content (glucosamine addition). In HAS2 transfected cells, glucosamine treatment tended to cause a reduction in hyaluronan coat, but the response was highly variable and statistically not significant (Fig. 4B).
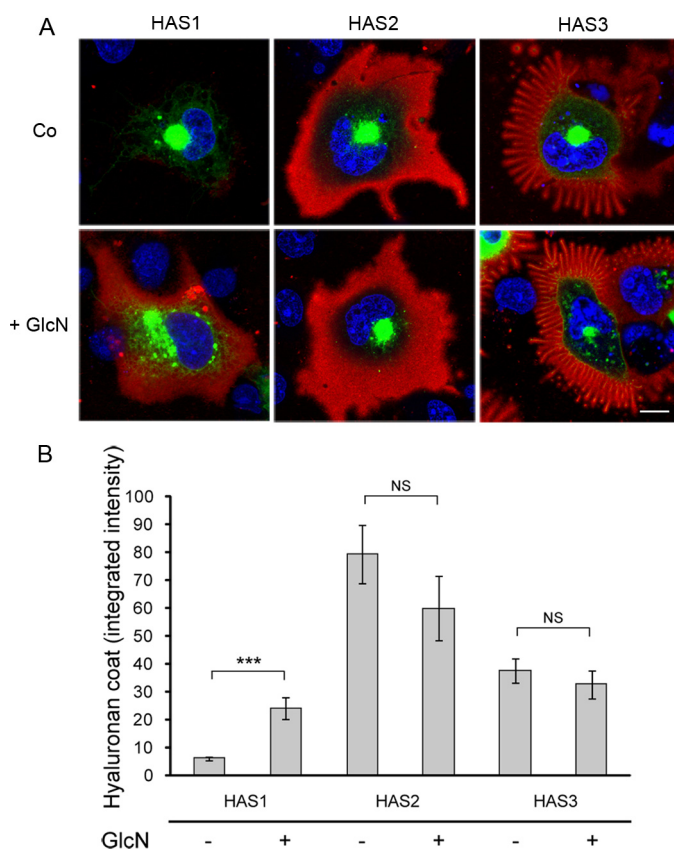
A

B

FIGURE 4. **Cell surface hyaluronan coats on COS-1 cells expressing different HAS isoenzymes in the presence and absence of 1 mM glucosamine.** *A*, confocal optical sections through Dendra-*Has*-transfected live COS-1 cells were taken after 6 h with (+ *GlcN*) and without (control, *Co*) 1 mM glucosamine. *Green*, Dendra2-HAS; *red*, hyaluronan coat; *blue*, nuclei. *B*, the mean size of the hyaluronan coat per cell was quantified with image analysis (mean intensity $\times$ area of the fluorescent HABC signal per cell). The means $\pm$ S.E. of four independent experiments, each including 10–171 cells for each treatment picked at random, are shown. The significance for glucosamine treatment as compared with control is shown. ***, $p < 0.001$; *NS*, not significant, by *t* test.

In contrast to *Has2* and *Has3*, cells transfected with *Has1* showed almost no cell surface hyaluronan, a finding in line with the above notion that *Has1* transfection resulted in no increase in hyaluronan synthesis. However, supplementation of the cultures with 1 mM glucosamine produced a strong coat also on *Has1*-transfected cells (Fig. 4*A*). Assay of the hyaluronan-associated fluorescence per transfected cell suggested that in the presence of glucosamine, the size of the *Has1* coat approached that on *Has3*-transfected cells (Fig. 4*B*), indicating that HAS1 is capable of creating a hyaluronan coat but only in the presence of generous amounts of the UDP-sugars.

Effect of Glucose on Hyaluronan Synthesis by HAS1–3— UDP-GlcNAc and UDP-GlcUA are both downstream metabolites of glucose, the availability of which is therefore supposed to influence cellular content of these substrates and the ability of HASs to produce hyaluronan. The standard culture medium of COS-1 cells contained 25 mM glucose. When transferred in a glucose-free medium, a sharp decline in the cellular content of UDP-GlcNAc and UDP-GlcUA was found, whereas glucose concentrations between 5 and 25 mM did not differ from each other, suggesting a regulation that maintains these levels despite oversupply of glucose (Fig. 5, *A* and *B*).

Raising glucose concentration from 0 mM to a physiological (5 mM) level increased hyaluronan in the medium by 2.5-, 3.6-, and 2.4-fold in cultures transfected with *Has1*, *Has2*, and *Has3*, respectively (Fig. 5, *D–F*), results corresponding to those of UDP-sugars. Interestingly, despite little change in UDP-sugars levels between 5 and 25 mM glucose concentrations, hyaluronan production was increased by 96, 36, and 55% with HAS1, 2, and 3, respectively (Fig. 5, *D–F*). The low basal level of hyaluronan in the mock transfected cultures showed an increasing trend in the absence of glucose (Fig. 5*A*), a notion again in line with a feedback regulation of endogenous *Has2* expression by UDP-GlcNAc (supplemental Fig. S2) (34).

In the total absence of glucose (0 mM), even HAS2- and HAS3-overexpressing cells showed significantly attenuated hyaluronan coats (Fig. 6), whereas their coat sizes were not markedly changed between 5 and 25 mM glucose concentrations (Fig. 6*B*). HAS1 produced virtually no hyaluronan coat at any glucose concentration. These results indicate that a physiological glucose concentration is necessary for hyaluronan production even for HAS3, the isoenzyme least sensitive to UDP-sugar content, whereas increasing glucose beyond this level produces a relatively smaller further enhancement in hyaluronan synthesis.

*Effect of Stimulated Hyaluronan Synthesis on UDP-Sugar Pool Size—*UDP-sugars are produced in the cytosol and pumped by high affinity transporters into Golgi apparatus, the main site of glycosyl transferases and a privileged site of cellular glycosylations (31). Hyaluronan synthesis uses the cytosolic pool of the UDP-sugars and therefore faces first any deficiency in their availability. On the other hand, active hyaluronan synthesis is supposed to consume a fair amount of these substrates, and it is not known whether this is important in the cellular scale of their production capacity and whether the flux into hyaluronan is reflected in their content.

To allow hyaluronan production also by HAS1, the influence of HAS activity on UDP-sugar content was studied in the presence of 1 mM glucosamine. A significant depletion of both UDP-GlcNAc and UDP-GlcUA was found in cultures overexpressing HAS3, and a similar trend was seen with HAS1 and HAS2 (Fig. 7). In conclusion, hyaluronan synthesis can spend an important proportion of UDP-GlcNAc and UDP-GlcUA, which need to be continuously replenished and the scarcity of which may influence the synthesis of hyaluronan itself and other glycosylations dependent on the cytosolic content of these metabolites.

*Content of UDP-HexNAc and UDP-GlcUA in Different Cell Types—*The data above suggested that even if large amounts of HAS1 would be naturally expressed in COS-1 cells, it would not be able to synthesize hyaluronan because of the low supply of the UDP-sugars. To check whether a similar, relative shortage of the HAS substrates is more widespread, we analyzed the contents of UDP-sugars in other cell types.

Approximately 50-fold differences were found between different human cell types in the content of UDP-HexNAc and UDP-GlcUA (Fig. 8, *A* and *B*). The contents of the two UDP-sugars were also correlated with each other, suggesting that they are coregulated in a way that supports hyaluronan synthesis, requiring both substrates (Fig. 8, *A* and *B*). The content of
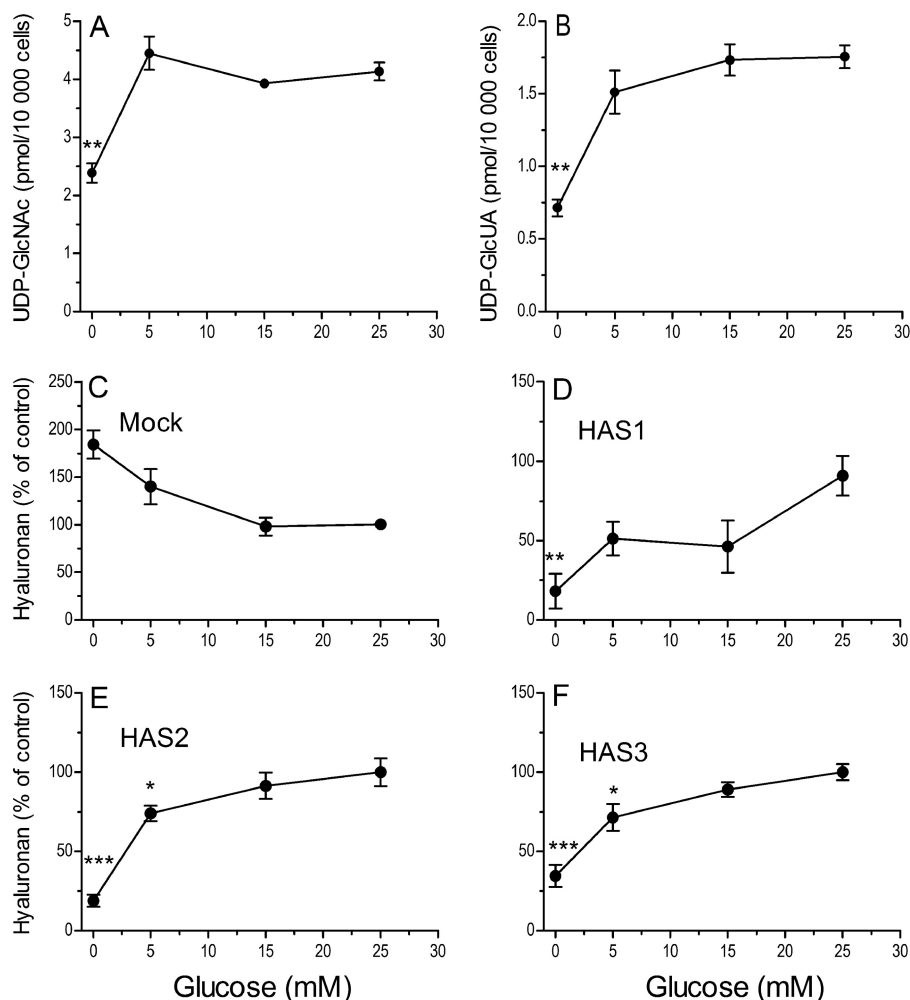
FIGURE 5. **UDP-sugar contents and hyaluronan synthesis in *Has1–3*-transfected COS-1 cells cultured in different glucose concentrations.** *A* and *B*, the cellular contents of UDP-HexNAc and UDP-GlcUA were determined from COS-1 cells grown on medium containing 0, 5, 15, and 25 mM glucose, which is the norm, for 6 h. *C–F*, hyaluronan was assayed from the medium of *Has1–3* and mock transfected COS-1 cells grown in the same glucose concentrations. The means ± S.E. of three (UDP-sugar levels) or four (hyaluronan) independent experiments, each with two replicates, are presented. Reduction of glucose significantly affected hyaluronan production by all three HASs ($p = 0.014$ for HAS1 and $p < 0.001$ for HAS2 and HAS3, measured by ANOVA). The UDP-GlcNAc and UDP-GlcUA levels were also significantly affected by glucose ($p = 0.0013$ for UDP-GlcNAc and $p = 0.0016$ for UDP-GlcUA, measured by ANOVA). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, as compared with 25 mM glucose by Dunnett's post hoc test.

the UDP-sugars was high in the cells of mesenchymal origin, *i.e.*, in different fibroblasts, and particularly high in mesothelial cells (Fig. 8, *A* and *B*).

The contents of UDP-GlcNAc and UDP-GlcUA required by HAS1 to produce hyaluronan in COS-1 cells, ~40 and 5 pmol/10,000 cells, respectively (Figs. 2 and 3), were exceeded in other cell types except keratinocytes and chondrosarcoma cells (Fig. 8, *A* and *B*). Because the range of hyaluronan quantities produced by different cells is also extensive, we compared the levels of the two UDP-sugars with hyaluronan secreted in the culture medium. In the cell types studied, the rate of hyaluronan synthesis varied between 1 and 500 ng/10,000 cells during a 24-h incubation (Fig. 8*C*). A high level of hyaluronan was associated with high contents of UDP-HexNAc and UDP-GlcUA (Fig. 8, *A–C*). Thus, cell types producing large quantities of hyaluronan were also armed with a larger pool of the UDP-sugars.

Relationship between UDP-Sugar Content and HAS1–3 Expression—The levels of *Has* enzymes expressed in the set of cells analyzed for the UDP-sugars and hyaluronan production were quantified by real time RT-PCR. The highest variation

between cell types was found in *Has1* (~10,000-fold), followed by *Has2* (~200-fold), with *Has3* showing least variation (~10-fold). Interestingly, the expression of *Has1* showed very strong correlation with the cellular levels of both UDP-HexNAc and UDP-GlcUA ($r = 0.978$ and 0.997, respectively) (Fig. 9, *A* and *B*). The mRNA level of *Has2* was also correlated with UDP-HexNAc and UDP-GlcUA ($r = 0.892$ and 0.815, respectively; Fig. 9, *C* and *D*), whereas no correlation was found between *Has3* expression and UDP-sugar contents (Fig. 9, *E* and *F*). These data strongly suggest that the expression of the *Has1* isoenzyme, most dependent on high UDP-sugar contents, is coordinated with a metabolic state that maintains a high level of these substrates.

DISCUSSION

The present data indicate that the three HAS isoenzymes have widely variable requirements for the cellular supply of UDP-GlcNAc and UDP-GlcUA *in vivo*. HAS1 seems to produce little if any hyaluronan in cells with a low content of the nucleotide sugars, whereas HAS3 can do it almost irrespective
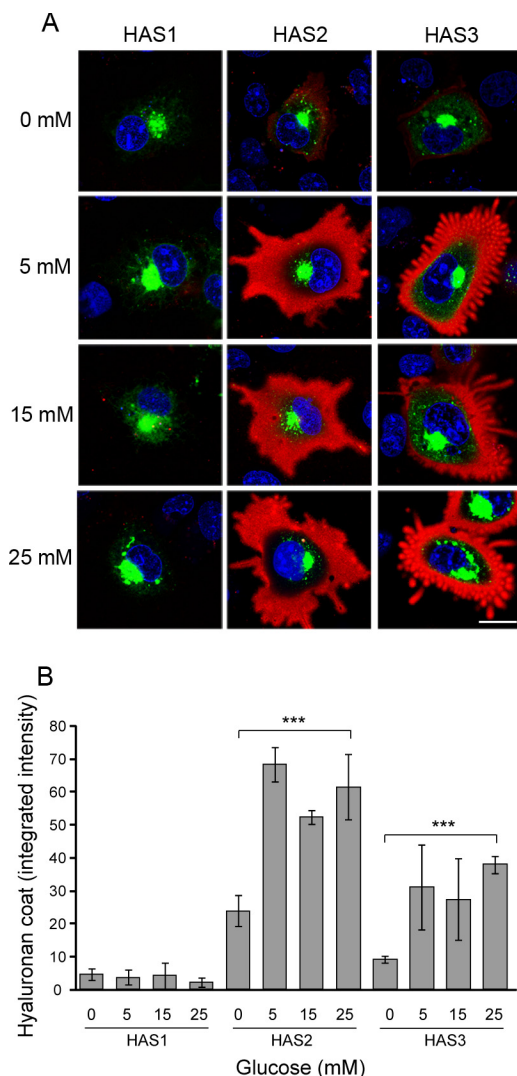
FIGURE 6. **Glucose concentration effects on hyaluronan coats in *Has1–3* transfected cells.** Hyaluronan coats were analyzed with confocal microscopy in individual live cells expressing HAS1–3 after 6 h of incubation in 0–25 mM glucose. Examples of cells are shown in *A*, and the integrated intensity of the coats was determined with image analysis as shown in *B*. Green, Dendra2-HAS; *red*, hyaluronan coat; *blue*, nuclei. The means and ranges of two independent experiments, each including analysis of 16–69 cells picked at random, are shown in *B* ($p < 0.001$, measured by ANOVA). ***, $p < 0.001$ as compared with 25 mM glucose by Dunnett's post hoc test.
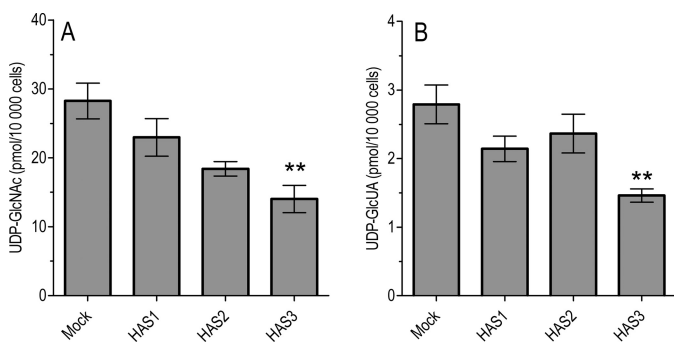

FIGURE 7. **Effect of *Has1–3* transfections on cellular UDP-sugars.** HAS1–3-expressing COS-1 cells cultured for 6 h in media supplied with 1 mM glucosamine were analyzed for the content of UDP-HexNAc (*A*) and UDP-GlcUA (*B*). The means ± S.E. of four separate experiments are shown. Both UDP-GlcNAc and UDP-GlcUA were significantly affected by HAS3 expression, $p = 0.0053$ and 0.0042, respectively as tested by ANOVA. **, $p < 0.01$ as compared with mock transfection by Dunnett's post hoc test.

of the endogenous levels of these substrates. Furthermore, the contents of these nucleotide sugars greatly vary between cell types and strongly correlate with the expression level of *Has1*, suggesting that the differentiation state of the cell coordinates the expression of this *Has* isoenzyme.

Transfections, HAS Expressions, and Hyaluronan Secretion—Most cell types analyzed so far express more than one *Has*, with each isoenzyme at different level and with different response to external signals. This makes it difficult to study the distinct properties of each HAS in live cells. In the present work, we aimed at avoiding the confounding contribution of the three genes to hyaluronan synthesis by overexpressing each HAS in COS-1 cells known for their minimal hyaluronan synthesis. The transfected genes were also beneficial in that their promoters were unresponsive to endogenous regulatory signals that may cause uncontrollable changes in the expression level. Transient transfections were considered preferable because approximately equal HAS protein expression levels per cell were obtained, and the risk of changes in the cellular background during selection of stable transfectants was avoided.

The fact that the transfected genes were not expressed in all cells (transfection efficiency, <100%) means that some of the effects were "diluted." These effects include the magnitude of the changes of hyaluronan content in the growth medium of cultures with variable glucosamine (Fig. 3) and glucose concentrations (Fig. 6C) and the degree of UDP-sugar depletion during stimulated hyaluronan synthesis (Fig. 7). Therefore, both hyaluronan and UDP-sugar changes were actually greater than those detected. This dilution was avoided in the microscopic assays that specifically examined cells expressing the HAS genes, and the data completely supported the biochemical assays.

UDP-Sugar Requirements of HAS1–3—The finding that efficient hyaluronan synthesis requires higher cellular UDP-GlcNAc and UDP-GlcUA concentrations in the order HAS1 > HAS2 > HAS3 is in agreement with a previous *in vitro* study on membrane preparations of rat 3Y1 cell homogenates (16). In low UDP-GlcUA concentrations, the reported K_m values of HAS1, HAS2, and HAS3 for UDP-GlcNAc are 799, 108, and 82, respectively (16), *i.e.*, there is ~10-fold difference between HAS1 and HAS3. The enigma of HAS1 transfection producing little or no hyaluronan has been recognized before in COS-1 cells (11) and can probably be explained by differences in the supply of UDP-GlcNAc.

The K_m values for UDP-GlcUA are up to ~10-fold lower than those for UDP-GlcNAc and show considerably less variation between HAS isoenzymes (K_m = 53–73, 30–33, and 34–35 in HAS1, HAS2, and HAS3, respectively) (16). Therefore, UDP-GlcUA does not discriminate HAS enzymes from each other as much as UDP-GlcNAc (16). In general, the present work demonstrates that the different requirements of the HAS isoenzymes for UDP-GlcNAc are likely to have biological importance because the UDP-sugar contents greatly vary between cell types.

UDP-Sugar Drain into Hyaluronan—The present results show that the high expression of HAS obtained by transfection can exceed the cellular capacity of UDP-GlcNAc and UDP-GlcUA production, as indicated by the depletion of the UDP-
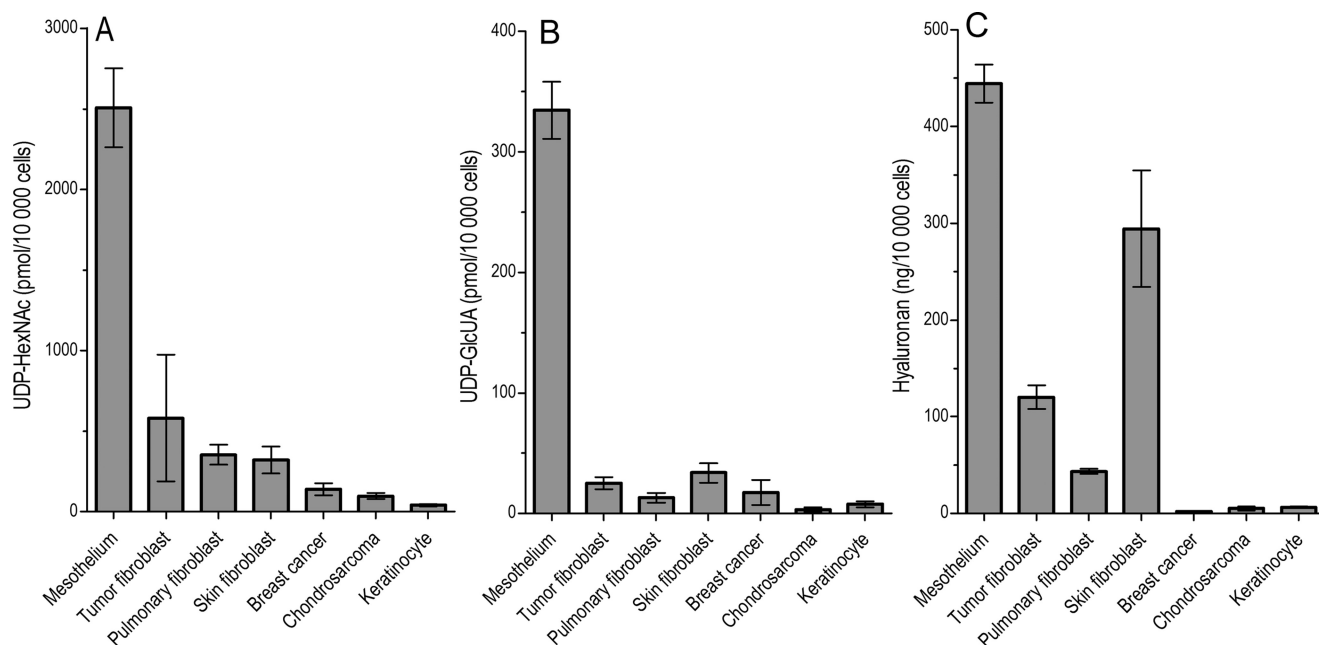
FIGURE 8. **UDP-sugars and hyaluronan synthesis in different human cell types.** Cellular UDP-HexNAc (*A*) and UDP-GlcUA contents (*B*) and hyaluronan (*C*) in the growth medium were quantified in mesothelial cells (LP-9), fibroblasts from squamous cell tumors, pulmonary fibroblasts (WI-38), skin fibroblasts, breast cancer cells (MCF-7), chondrosarcoma cells, and epidermal keratinocytes (HaCaT). The means ± S.D. of three replicates are shown.

sugars. Equally high rates of hyaluronan synthesis exist also naturally in some cell types, suggesting that a significant part of the UDP-sugars can end up in hyaluronan. Without any replenishment, the normal UDP-GlcNAc pool of COS-1 cells (4 pmol/10,000 cells) would be enough to synthesize eight times the small amount of hyaluronan naturally secreted per 24 h (0.5 pmol of disaccharide/10,000 cells). However, following HAS3 transfection, more than 10 times the steady state pool size of UDP-GlcNAc is needed for daily hyaluronan production. Because of its lower content and perhaps fewer natural targets of use, the proportion and rate of UDP-GlcUA flux to hyaluronan are likely to be even larger and faster than that of UDP-GlcNAc.

In addition to hyaluronan synthesis, cytosolic UDP-GlcNAc goes into building the dolichol-phosphate oligosaccharides that form the core of the *N*-linked oligosaccharides (32) and is used by the enzyme *O*-GlcNAc transferase to couple a single GlcNAc residue to serine and threonine residues of cytosolic and nuclear proteins for signaling purposes (33). It was recently shown that cellular UDP-GlcNAc down-regulates transcription of HAS2 (34) but increases the stability of HAS2 protein (35). In contrast, no *O*-GlcNAc-dependent regulation was found in HAS3 (35). *O*-GlcNAc modification of HAS1 or its regulatory protein could also influence its enzymatic activity. If this would be the case, the regulation works at a considerably higher range of UDP-GlcNAc levels than that of HAS2, and the difference in activity and UDP-GlcNAc dependence to the HAS2 and HAS3 persists.

Depletion of cellular UDP-GlcNAc by various means has been repeatedly shown to lower the level of *O*-GlcNAc modification of various proteins (36, 37). Interestingly, if endogenously increased hyaluronan synthesis also depletes the UDP-sugar pools, it could have widespread consequences.

Hyaluronan Synthesis and Glucose Supply—All HAS isoenzymes produced considerably lower amounts of hyaluronan when cultured in the absence of glucose, as compared with concentrations close to physiological level (5 mM). Although UDP-sugar contents in nontransfected cultures remained unchanged between 5 and 25 mM glucose (Fig. 5, *A* and *B*), a stimulation of hyaluronan synthesis was observed in cells expressing extra HAS. This seeming discrepancy was likely due to the fact that the synthesis of hyaluronan decreased the amounts of the UDP-sugars (Fig. 7), and the higher glucose concentration in medium helped the cells to keep up with the higher demand and better support HAS activity.

Although the levels of UDP-sugars have not been assayed, there are several examples of cells which, when subjected to "diabetic" glucose concentrations, show changes consistent with increased cellular UDP-GlcNAc supply and hyaluronan synthesis. High glucose concentrations result in increased hyaluronan production in mesangial cells (38), renal proximal tubular cells (39), renal interstitial fibroblasts (40), and vascular smooth muscle cells (41, 42). Hyaluronan cumulates in the glomeruli of diabetic animals (43), and hyperglycemia induces mesangial cells to synthesize a hyaluronan coat that can recruit inflammatory cells (44, 45). Actually, HAS1 expression levels are induced with factors involved in inflammation, like IL-1 β in uterine fibroblasts (46), TGF-β in mesothelial cells (9), and prostaglandins in synoviocytes (47), indicating an important role for HAS1 in conditions associated with abnormal glucose metabolism. Serum hyaluronan level is higher in diabetic patients than in healthy subjects and correlates with poor glucose control in the patients (48). Therefore, increased hyaluronan secretion in response to raised glucose levels has been suggested to contribute to diabetic pathologies, including inflammation.
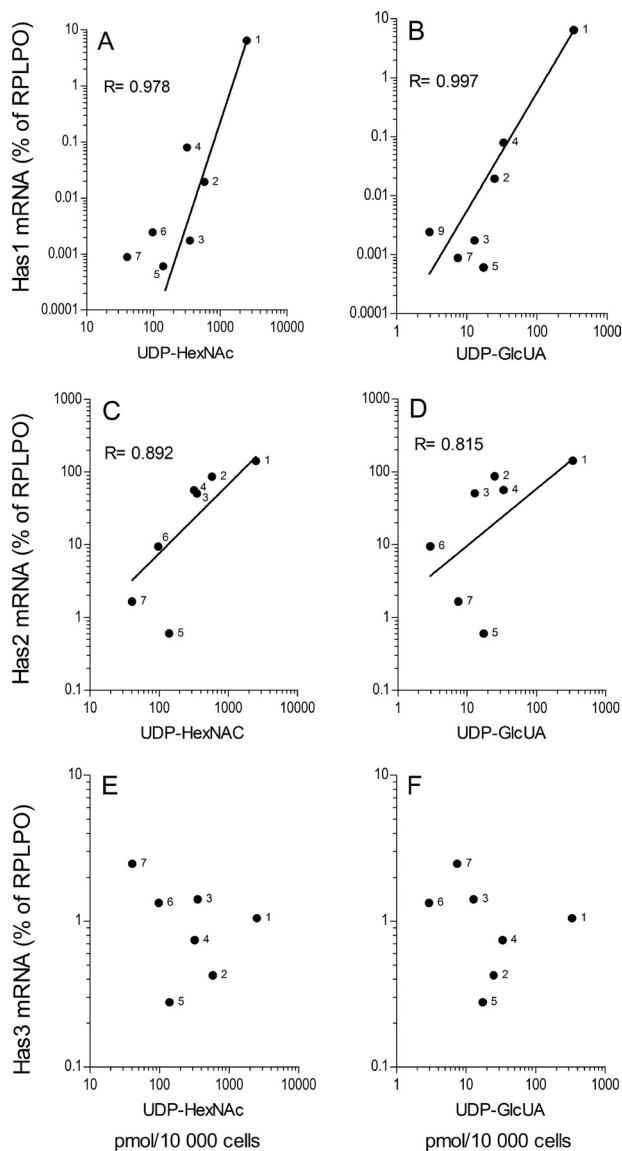
FIGURE 9. **Correlations between the expression of *Has1–3* and cellular contents of UDP-sugars in different human cells types.** The mRNA levels of *Has1–3* were determined in the cell types listed in Fig. 8 by real time RT-PCR using *RPLPO* for normalization. UDP-HexNAc (*A, C, E*) and UDP-GlcUA (*B, D, F*) levels were determined from the same cell lines. Pearson correlations were calculated for all. *A* and *B*, $p = 0.000$. *C*, $p = 0.007$. *D*, $p = 0.026$. *Point 1*, mesothelial cell; *point 2*, tumor fibroblast; *point 3*, pulmonary fibroblast; *point 4*, skin fibroblast; *point 5*, breast cancer cell; *point 6*, chondrosarcoma cell; *point 7*, keratinocyte.

Likewise, rapidly growing malignant cells have glucose uptake and glycolysis rates usually higher than those of their normal tissues of origin (49). The increased glucose metabolism may, through increased UDP-GlcNAc content, contribute to the hyaluronan deposits found in most cancers (50). Excessive hyaluronan promotes cancer growth and is one of the novel targets of therapy (51). In summary, it is highly likely that many pathological reactions are eventually initiated or supported by an enhanced UDP-GlcNAc supply derived from glucose. Therefore, it is important to learn more about the regulation of cellular metabolism of UDP-sugars and the specific implications for hyaluronan synthesis by the different HAS enzymes.

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