Plasma Membrane Residence of Hyaluronan Synthase Is Coupled to Its Enzymatic Activity*§

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Hyaluronan is a multifunctional glycosaminoglycan up to $10^7$ Da molecular mass produced by the integral membrane glycosyltransferase, hyaluronan synthase (HAS). When expressed in keratinocytes, N-terminally tagged green fluorescent protein-HAS2 and -HAS3 isoenzymes were found to travel through endoplasmic reticulum (ER), Golgi, plasma membrane, and endocytic vesicles. A distinct enrichment of plasma membrane HAS was found in cell protrusions. The total turnover time of HAS3 was 4–5 h as judged by the green fluorescent protein signal decay and hyaluronan synthesis inhibition in cycloheximide-treated cells. The transfer from ER to Golgi took about 1 h, and the dwell time on the plasma membrane was less than 2 h in experiments with a relief and introduction, respectively, of brefeldin A. Constructs of HAS3 with 16- and 45-amino-acid C-terminal deletions mostly stayed within the ER, whereas a D216A missense mutant was localized within the Golgi complex but not the plasma membrane. Both types of mutations were almost or completely inactive, similar to the wild type enzyme that had its entry to the plasma membrane experimentally blocked by brefeldin A. Inhibition of hyaluronan synthesis by UDP-glucuronic acid starvation using 4-methyl-umbelliferone also prevented HAS access to the plasma membrane. The results demonstrate that 1) a latent pool of HAS exists within the ER-Golgi pathway; 2) this pool can be rapidly mobilized and activated by insertion into the plasma membrane; and 3) inhibition of HAS activity through mutation or substrate starvation results in exclusion of HAS from the plasma membrane.

Hyaluronan is a high molecular mass, unbranched acidic glycosaminoglycan found in the extracellular matrix of many vertebrate tissues. This glycosaminoglycan contributes to differentiation, development, and repair functions of several cell types and tissues, and its absence results in lethality during embryogenesis due to multiple developmental defects including the failure of endocardial cushion formation (1).

Hyaluronan synthases (HASs) are unique enzymes that synthesize hyaluronan from UDP-activated N-acetylgalcosamine and glucuronic acid and coordinately translocate the growing chain through the plasma membrane (2). Three different isoenzymes exist in mammals (3) and Xenopus (4). Certain pathogenic strains of Streptococci and Pasteurella bacteria also possess hyaluronan synthases with some sequence homology to the vertebrate enzymes but utilize different enzymatic mechanisms. The vertebrate and Pasteurella enzymes add the alternating hexose moieties to the non-reducing end of the chain, whereas the streptococcal enzyme elongates hyaluronan from the reducing end (5, 6). The enzyme from Pasteurellae appears to have separate domains for the transfer of the two precursors, whereas the transfer functions of the streptococcal and vertebrate enzymes appear more integrated. At least one vertebrate hyaluronan synthase (HAS1) can, however, also synthesize short homo-oligomers of N-acetylgalcosamine (chitin) in vitro (7, 8). The relative enzymatic rates and the ultimate hyaluronan size may also differ between the isoenzymes (9–11).

Hyaluronan synthases are integral membrane proteins with an active site located in the cytoplasmic side of the plasma membrane (2). In this respect, hyaluronan differs from other complex carbohydrates, the synthesis of which begins in the endoplasmic reticulum and is completed in the Golgi apparatus. Hyaluronan synthases do not have a typical signal sequence at their N terminus, suggesting that they may not be processed via the usual secretory pathway through the Golgi complex. Also, HAS enzymes have not been found to undergo any post-translational modifications that often take place in the Golgi. Furthermore, brefeldin A, which blocks the Golgi plasma membrane traffic, had no effect on hyaluronan secretion in chondrocytes even after a prolonged incubation (12, 13), suggesting either that the HASs have a long half-life or that they are not processed through the Golgi pathway. However, it was recently shown that green fluorescent protein (GFP)-tagged Xenopus Has1 became co-translationally inserted into endoplasmic reticulum (ER) and traveled through the Golgi on its way to the plasma membrane (14). Irrespective of the route to the plasma membrane, no data are currently available to indicate that any HAS synthesizes hyaluronan before reaching the cell surface.

The low expression level of HASs in normal keratinocytes, and in many other cell types, has impeded microscopic studies of their localization and interactions with other molecules. Development of antibodies suitable for specific immunocyto-
chemical detection of the relatively scarce HAS proteins has not succeeded so far. Therefore, expression of enzymatically active HASs as GFP-tagged fusion proteins provides an opportunity to 1) localize the enzymes directly in living cells, 2) follow their movements subcellularly, 3) examine possible regulatory points in their ability to synthesize hyaluronan, and 4) detect the influence of elevated hyaluronan synthesis rate on important cellular functions, such as those described previously in keratinocytes (15).

In the present study, we performed high resolution confocal microscopy on epidermal keratinocytes expressing GFP-labeled mouse HAS polypeptides. Colocalization experiments showed strong labeling in the Golgi and trans-Golgi network, in addition to the plasma membrane and endocytic vesicles. Experimental inhibition of vesicular traffic through the Golgi to the plasma membrane reduced the residence of GFPHAS on the plasma membrane and inhibited hyaluronan synthesis, suggesting that HAS trafficking proceeds through this organelle. Furthermore, an enzymatically inactive, missense mutant and C-terminally truncated GFPHAS3 fusion proteins were not detected on the plasma membrane. Likewise, depletion of the UDP-hexose precursor pools prevented the localization of intact HAS on the plasma membrane. It thus seems that ongoing hyaluronan synthesis is required for HAS residence on the plasma membrane.

MATERIALS AND METHODS
Keratinocyte Cultures—A newborn rat epidermal keratinocyte cell line (REK) (16) was cultured in minimum essential medium (Invitrogen) supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 4 mM glutamine (Sigma), and 50 μg/ml streptomycin sulfate and 50 units/ml penicillin (Sigma). Keratinocytes were passaged twice a week using 0.1% trypsin (v/v) in 0.02% EDTA (Sigma) in phosphate-buffered saline (Regaen Ltd, Kuopio, Finland). In some experiments, the synthesis and secretory pathways were manipulated by adding brefeldin A (5 μg/ml, Sigma), cycloheximide (50 μg/ml) (18) in 1% bovine serum (Hyclone), or nocodazole (25 μM, Sigma) in the culture medium for the times indicated in the figure legends. To localize endocytosed material, Alexa Fluor® 594 Hydrazide (0.8 μM, Molecular Probes, Eugene, OR) was added to the culture medium.

Transfections—Cells were routinely plated at a density of 0.1 x 10⁴ cells/well on collagen-coated 8-well chambered cover glasses (Nalge Nunc, Naperville, IL). When subconfluent, the cells were transiently transfected with mammalian expression vectors encoding mouse Has2 or Has3 open reading frames that had been fused in-frame at their N termini with the enhanced GFP sequence (17) using FuGENE 6 transfection reagent (Roche Applied Science). In some experiments, GFP-Has3 containing an inactivating missense mutation (D216A) or C-terminal truncations (R539stop and E510stop, respectively) were used. These constructs were generated through site-directed mutagenesis of Has2 and Has3 open reading frames with N-terminally fused GFP were expressed in rat epidermal keratinocytes. To show that the transfected GFPHASs were functional in the REK cells, the amount of hyaluronan released into growth medium was measured using an ELSA assay (Fig. 1). The content of hyaluronan in the medium was clearly higher in the cultures transfected with GFP-Has2 and GFP-Has3 constructs than in those transfected with the empty GFP vector (Fig. 1). The proportion of transfected cells varied from 10 to 50% between different experiments (visual evaluation and fluorescence-activated cell sorter analysis, data not shown), indicating that the synthesis of hyaluronan in the cells transfected was 10–30 times elevated as compared with non-transfected cells. There were no consistent differences in the expression rate or hyaluronan synthesis between GFP-Has2 and GFP-Has3.

RESULTS
Transfection of Functional GFP-HAS2 and -HAS3 Constructs in REK Cells—Expression plasmids containing mouse Has2 and Has3 open reading frames with N-terminally fused GFP were expressed in rat epidermal keratinocytes. To show that the transfected GFPHASs were functional in the REK cells, the amount of hyaluronan released into growth medium was measured using an ELSA assay (Fig. 1). The content of hyaluronan in the medium was clearly higher in the cultures transfected with GFP-Has2 and GFP-Has3 constructs than in those transfected with the empty GFP vector (Fig. 1). The proportion of transfected cells varied from 10 to 50% between different experiments (visual evaluation and fluorescence-activated cell sorter analysis, data not shown), indicating that the synthesis of hyaluronan in the cells transfected was 10–30 times elevated as compared with non-transfected cells. There were no consistent differences in the expression rate or hyaluronan synthesis between GFP-Has2 and GFP-Has3.

We also transfected a GFP-Has3 construct with a missense mutation in the enzyme active site (D216A), and we transfected two truncated forms, lacking 16 and 45 amino acids from the C terminus (R539stop and E510stop, respectively). In cultures transfected with these mutated GFP-Has3-forms, the amount of secreted hyaluronan was close to the level of control cultures transfected with GFP only (Fig. 1).

Distribution of GFP-HAS2 and HAS3 in REKs—Both GFPHAS2 and GFPHAS3 were abundant inside the cells, forming vesicular structures often grouped around the nucleus and morphologically resembling the ER-Golgi complex (Fig. 2, A–E, arrowheads) (videos 1 and 2, supplemental material). The
GFP-HAS Localization

FIG. 1. Hyaluronan secretion of keratinocytes transfected with constructs expressing GFP-HAS fusion proteins. REKs were transiently transfected with wild type GFP-Has2- and GFP-Has3 constructs or with GFP-Has3 constructs with a missense mutation (D216A) or truncation at the C terminus (R539Stop and E510Stop). Culture medium from a 24-h period was collected, and the amount of secreted hyaluronan was quantitated by an ELSA assay. The results represent one of four experiments with similar results. Error bars indicate ranges of duplicate wells.

FIG. 2. Localization of GFP-HAS3 and GFP-Has2 fusion proteins in transiently transfected keratinocytes. A, B, and D, HAS3-transfected cells; C and E, HAS2-transfected cells; F, GFP control transfected cell. Living cells were imaged using laser confocal microscope. The images in A, B, C, and F and the inset are single optical sections through the cells, whereas D and E are z-sections obtained from primary image stacks composed of horizontal optical sections through the cells. Small arrows indicate the location of GFP signal on the plasma membrane projections, large arrows point to the circular signal in the intracellular vesicles, and arrowheads show Golgi-like vesicles. Magnification bars represent 10 µm.

GFP-Has2 and GFP-HAS3 were found on the plasma membranes (Fig. 2, B and C, small arrows), although GFP-Has3 showed more intense and widely spread cell surface signal than GFP-Has2. GFP-Has2, and particularly GFP-HAS3, were abundant on filopodial extensions and especially enriched in their tips (Fig. 2, A–C, and inset, small arrows) (videos 1 and 2, supplemental material). The GFP-HAS3 decoration on the small dorsal plasma membrane projections was particularly obvious in the three-dimensional reconstruction (Fig. 2D). The empty GFP control showed a diffuse cytoplasmic staining (Fig. 2F).

There were often small, labeled vesicles in the peripheral cytoplasm (Fig. 2, B–E), and some GFP-Has3-transfected cells also contained larger vesicles, structures resembling endocytic vesicles, with a circular, GFP-positive signal (Fig. 2B, large arrows) (video 1, supplemental material). The GFP-labeled HAS proteins thus appeared to exist throughout the secretory route, including ER, Golgi complex, plasma membrane, and endocytic vesicles, with selective enrichment in membrane projections on the dorsal cell surface.

GFP-HAS Colocalization with Subcellular Markers—Anti-CD44 immunostaining did not label any intracellular structures but decorated the cell surface, co-localizing with GFP-Has3 and confirming its plasma membrane localization (Fig. 3A). The perinuclear GFP-Has3 showed partial colocalization

FIG. 3. Co-localization analysis of GFP-HAS3 within the cells. Fixed GFP-Has3-transfected cells were stained with an anti-CD44 antibody (A), anti-58K antibody (B and D), and anti-calnexin antibody (C) to visualize plasma membrane, Golgi apparatus, and ER, respectively, using Texas Red-labeled secondary antibodies. In D, the cells were treated with nocodazole (25 µM, 2 h) before fixation to break down the microtubules. In E, living GFP-HAS3-expressing keratinocytes were incubated for 30 min at 37 °C with Alexa Fluor® 594 hydrazide (red), a marker for fluid phase endocytosis. In F–L, GFP-Has3-transfected REKs were stained with bHABC to localize hyaluronan using TR-labeled streptavidin as a secondary reagent. The total cell-associated hyaluronan is shown in F, whereas in G–I, the pericellular hyaluronan was removed by digesting fixed cells with Streptomyces hyaluronidase before permeabilization to show the intracellular hyaluronan. To reveal whether the intracellular hyaluronan originated from extracellular sources, Streptomyces hyaluronidase was added to the growth medium of live cell cultures for 30 min (J), 1 h (K), or 4 h (L) before termination of the culture. Single optical sections are shown in A–E and in H and I, and compressed image stacks of optical sections are shown in F and G and in J–L. The inset in F shows the localization of hyaluronan on the top of GFP-Has3-positive filopodia. The arrowheads in E, H, and I indicate the intracellular HAS3 vesicles containing hyaluronan. Magnification bars, 10 µm.
with the 58K Golgi marker (Fig. 3B) and a trans-Golgi marker (data not shown). The ER marker calnexin also showed partial colocalization with GFP-HAS3 in vesicles close to the nucleus, although most of the ER marker resided more peripherally in the cytoplasm and displayed little colocalization with GFP-HAS3 (Fig. 3C). These colocalization experiments were also performed using GFP-Has2-transfected cells, which showed patterns essentially equivalent to those of GFP-Has3 (data not shown).

The association of the GFP-HASs with the Golgi elements of REKs was also studied by treating the cells with nocodazole, an agent known to disrupt the normal morphology of the Golgi complex (Fig. 3D). The morphology of the GFP-HAS3 structures was lost along with that of the Golgi apparatus, whereas many of the small vesicles resulting from Golgi dispersion retained the colocalization of 58K and GFP-HAS3 (Fig. 3D), supporting the idea that a significant proportion of GFP-HAS3 resided in the Golgi complex.

The morphology of some of the GFP-HAS3-positive vesicular structures suggested that they were derived from the plasma membrane (Fig. 2B), and time-lapse imaging showed trafficking of GFP-Has3 from cell surface to endosomes (video 1, supplemental material). A fluid phase marker added into growth medium (Fig. 3E) was rapidly taken up in a part of the smaller vesicles and most of the larger, circular GFP-HAS3-positive vesicles (Fig. 3E, arrowheads), suggesting that this GFP-HAS3 was derived through endocytosis from plasma membrane.

**Colocalization Analysis of Intracellular Hyaluronan and GFP-HAS3**—Despite the endogenous hyaluronan background, REKs transfected with GFP-Has3 and GFP-Has2 showed distinctively elevated levels of cell surface hyaluronan close to GFP-HASs (Fig. 3F), especially around the filopodia-like projections (Fig. 3F, inset). A portion of the hyaluronan resided within the cells, ascertained by removing pericellular hyaluronan with *Streptomyces* hyaluronidase digestion (Fig. 3, G–I). Fig. 3G shows the total amount of intracellular hyaluronan (a compressed stack of confocal images). A single optical section from the same cell is presented in Fig. 3H to reveal the actual, relatively minor colocalization of intracellular hyaluronan with GFP-HAS3. However, the extent of colocalization varied, as shown in an optical section from another cell with more substantial colocalization (Fig. 3I). In this cell, colocalization existed both in a portion of the small vesicles and in the large vesicles, resembling those positive for the tracer of endocytosis in Fig. 3E. The result indicates that hyaluronan and HAS3 can be co-endocytosed but does not exclude the possibility that a part of the vesicles are on their way from the Golgi apparatus to the plasma membrane.

The origin of the intracellular hyaluronan was studied by digesting extracellular hyaluronan with *Streptomyces* hyaluronidase in live cell cultures for up to 4 h prior to fixation and staining for hyaluronan (Fig. 3, J–L). As expected, pericellular hyaluronan was almost totally removed by the enzyme in 30 min (data not shown). After a 30-min digestion, the cells contained about equal, sometimes even enhanced, signal for intracellular hyaluronan, as compared with non-digested cells (Fig. 3J). However, a longer digestion (1–4 h) led to almost complete disappearance of the intracellular hyaluronan (Fig. 3, K and L), and the remaining hyaluronan showed no colocalization with intracellular GFP-HAS3. The data suggest that, initially, the partial degradation of cell surface hyaluronan enhanced its endocytosis and thus intracellular accumulation, whereas later on, the depleted hyaluronan pool on the cell surface reduced the flux into the cell, which, together with a constant decay of the endocytosed material, resulted in the disappearance of intracellular hyaluronan.

**Subcellular Localizations of Mutated HAS3**—Confocal analysis of REKs transfected with the point-mutated D216A and truncated R539stop and E510stop GFP-Has3 constructs showed that although the constructs were well expressed, their localizations markedly differed from those of the intact forms and also from each other. The truncated form R539stop (like E510stop, not shown) was mainly found in the peripheral cytoplasm, forming a reticular pattern (Fig. 4, A–C), with considerable colocalization with an ER marker (Fig. 4B), and to a lesser extent, with the Golgi marker (Fig. 4C). The point-mutated D216A, instead, was mainly localized in large vesicles adjacent to the nucleus, whereas a smaller proportion formed a reticular ER-like pattern (Fig. 4D). The D216A partially colocalized with the Golgi marker (Fig. 4F) and less with an ER marker (Fig. 4E). None of these modified GFP-HAS3 forms was clearly detectable on the plasma membrane, as confirmed by the absence of colocalization with CD44 (Fig. 4, G and H). The amount of cell-associated hyaluronan varied in these cells from low to moderate, being comparable with untransfected cells (data not shown). Hyaluronidase did not show any colocalization with the modified GFP-HASs (Fig. 4I). The results indicated that the C-terminally truncated HASs had difficulty in leaving the ER, whereas most of the missense mutated construct reached the Golgi complex but was not detectable on the plasma membrane.

**Effect of Brefeldin A and Cycloheximide on REK Hyaluronan Secretion**—To study the influence of HAS trafficking on hyaluronan synthesis, we treated REKs with brefeldin A, which interferes with vesicular traffic from the trans-Golgi network to the plasma membrane and redistributes Golgi elements back to ER (21). A 6-h treatment with brefeldin A caused a 60–80% reduction in the secretion of hyaluronan in the GFP-Has2- and -Has3-transfected cultures (Fig. 5, A and B). A similar degree of inhibition was also seen in cultures transfected with a vector...
containing only GFP and also in non-transfected cultures (Fig. 5A). The amount of hyaluronan released into the culture medium in cultures pretreated with brefeldin A for 2 h and then chased for 4 h varied from 70 to 95% of that both in control and in Has-transfected cultures (Fig. 5A), indicating that the effect of brefeldin A treatment was reversible.

Inhibition of protein synthesis using a 4-h cycloheximide treatment caused ~50% reduction in the amount of hyaluronan secreted into culture medium (Fig. 5B). When added together with brefeldin A, it did not further impair hyaluronan synthesis (Fig. 5B). To see whether the recovery of hyaluronan synthesis during the chase after brefeldin A treatment was due to newly synthesized HAS protein or redistribution of pre-existing HAS blocked in the Golgi and ER, we added cycloheximide into the chase medium of the cultures pretreated with brefeldin A. During the chase following brefeldin A block, hyaluronan secretion recovered almost at the same rate as in cultures chased without cycloheximide (Fig. 5B), suggesting that the recovery was largely due to HAS already existing in the secretory pathway.

**GFP-HAS3 Traffic in Brefeldin A and Cycloheximide-treated Cultures**—Confocal analysis showed that the GFP-HAS3 signal formed a diffuse reticular pattern in the cytoplasm of cultures treated with brefeldin A for 2 h (Fig. 6, A–C), resembling the images of the C-terminally truncated GFP-HAS3 (Fig. 4, A–C) and indicating that GFP-HAS3 had returned from the Golgi apparatus to ER. In the brefeldin A-treated cultures, no GFP-HAS3 was detected on the plasma membrane (Fig. 6A), also indicated by the lack of colocalization with CD44 (Fig. 6I). The GFP-HAS3 signal on the plasma membrane was often more intense in the chased than in the untreated control cultures, plasma membrane, and the large endocytic vesicles (Fig. 6F). The GFP-HAS3 signal on the plasma membrane was often more intense in the chased than in the untreated control cultures, and was close to strong hyaluronan staining, giving an impression of a rebound effect (Fig. 6F). Following release of the brefeldin A block, it thus took ~1 h to refill Golgi apparatus with GFP-HAS3 from ER, and the whole secretory and endocytosis pathway reached a new equilibrium by 2 h.

After a 30-min chase following brefeldin A block, most of the GFP-HAS3 signal still gave an ER-like pattern, with a smaller proportion on sites and structures corresponding to Golgi apparatus (Fig. 6D). A chase for 1 h brought most of the GFP-HAS3 to the Golgi complex and brought a small amount also to the plasma membrane (Fig. 6E). Cells harvested after a 2-h chase showed intense GFP-HAS3 labeling of Golgi-like structures, plasma membrane, and the large endocytic vesicles (Fig. 6F). The GFP-HAS3 signal on the plasma membrane was often more intense in the chased than in the untreated control cultures and was close to strong hyaluronan staining, giving an impression of a rebound effect (Fig. 6F). Following release of the brefeldin A block, it thus took ~1 h to refill Golgi apparatus with GFP-HAS3 from ER, and the whole secretory and endocytosis pathway reached a new equilibrium by 2 h.

In cells treated with cycloheximide for 2 h with a concentration that reduces protein synthesis by 95% in mouse keratinocytes (22), the distribution of GFP-HAS3 resembled that in the untreated cells (Fig. 6G). By 4 h, cycloheximide had reduced the signal, especially in the Golgi-like structures, whereas GFP-HAS3 was still found on the plasma membrane and small vesicles in the peripheral cytoplasm (Fig. 6H). After a 5-h
GFP Label as an Indicator for HAS Locations and Traffic Routes—The present results suggested that large proportions of total cellular GFP-HAS2 and GFP-HAS3 do not reside in their functionally active site on the plasma membrane. This was despite the deceptive impression of low GFP signal on the plasma membrane because of its spread over a large area, in contrast to the easily recognized, high signal density of the intracellular deposits. The intracellular localizations suggested that the GFP-HASs are en route through ER and Golgi complex, a conclusion in line with previous experiments using GFP-xlHAS1 in Vero cells (14).

It can be argued that either the GFP tag or the high expression level turns the protein to this route, perhaps not used with endogenous HAS. Although this alternative cannot be completely ignored, it becomes unlikely when considering the similar inhibition of the endogenous and transgene-driven hyaluronan synthesis under the influence of brefeldin A. If only overexpressed or GFP-HAS used the Golgi pathway, an inhibition by brefeldin A would not take place in non-transfected cells. Also, cycloheximide-induced inhibition of hyaluronan synthesis follows the same kinetics in non-transfected cells (23–26) and the GFP-Has-transfected cells in this work.

Our experiments in REKs with several polyclonal anti-HAS2 antibodies (gifts from Drs. John McDonald and Paraskevi Hel-din) supported the idea of a considerable intracellular pool of HAS since all of them show a signal in the perinuclear ER-Golgi area, in addition to the variable staining patterns in other parts of the cell (data not shown). It is also obvious that the GFP-tagged HASs are fully active in hyaluronan synthesis, supporting the idea of their correct processing and routing. There is thus nothing to suggest that the distribution or kinetics of HAS were altered by the GFP-tag.

C-terminal Truncation of Has3 and Exit from ER—The 45-amino-acid truncation at the C terminus of GFP-HAS3 (R539stop) made it enzymatically inactive in an in vitro synthase assay on membrane preparations, whereas a 16-amino-acid truncation (R539stop) left ~20% of the wild type activity,2 close to the ~13% seen here against the background of endogenous hyaluronan synthase in control REKs. The microscopy data indicated that the truncated proteins were mostly stuck in the ER, where a quality control system prevents the progress of defective proteins into Golgi apparatus. However, a minimal part of the HAS presumably resides in the luminal side of ER (27), where the misfolding recognition and chaperone functions work. It is also possible that the C terminus contains a cue to aid recruitment into vesicles transferring membrane material from ER to Golgi apparatus.

Golgi Complex Localization of HAS3 Point-mutated near the Catalytic Site—The GFP-HAS fusion protein with a missense mutation (D216A) in the large cytoplasmic loop of HAS3, presumably the domain important for catalytic activity (2), remained in the Golgi complex and perhaps recycled back to ER. This aspartate residue is conserved in all Family II glycosyltransferases and is required for their activity. The finding that D216A did not reach plasma membrane is somewhat surprising since the protein may remain properly folded and at least contains the parts necessary to cross the ER-Golgi boundary. Although the mutated amino acid may belong to a recognition site for recruitment to vesicles specifically destined for plasma membrane, this seems less probable than a hypothesis that the missing enzymatic activity as such is responsible for the inability of D216A to reach plasma membrane or stay there. The latter proposal is consistent with the finding that inhibiting hyaluronan synthesis by 4-MU reduced the residence of HAS3 on the plasma membrane. Perhaps whatever reason prevents HAS from synthesizing hyaluronan also prevents its association to the plasma membrane.

Disruption of Golgi Complex and HAS3 Entry to REK Plasma Membrane—On the membranes of the Golgi complex, brefeldin A prevents the assembly of coatomers necessary for the budding of transport vesicles toward plasma membrane and induces the return of Golgi material back to ER (28).

2 A. P. Spicer, unpublished data.
Accordingly, the transfer of GFP-HAS3 from the Golgi to the plasma membrane was blocked in REKs treated with brefeldin A. The GFP-HAS in the Golgi complex was apparently moved into ER (28), and that on the plasma membrane was apparently moved into endosomes (29). At the same time, the synthesis of hyaluronan was strongly inhibited. The present findings on HAS trafficking through the Golgi complex are consistent with those of GFP-xHas1-transfected to Vero cells (14) but different from the data on hyaluronan synthesis in chondrocytes, in which brefeldin A treatment up to 8 h did not prevent hyaluronan synthesis (12, 13). The content of hyaluronan in the culture medium was also reduced in the control, non-transfected cells, indicating that the difference to chondrocytes is not due to the transfection. The relatively rapid dislocation of GFP-HAS3 to ER and its depletion from plasma membrane suggested that the inhibition of hyaluronan synthesis is due to the block in HAS trafficking. The inconsistency in the influence of brefeldin A on hyaluronan synthesis between the cell types could be explained by a low HAS protein turnover on chondrocyte plasma membrane, but the other alternative, a direct transfer of the HAS from ER to the plasma membrane in chondrocytes, cannot be excluded at this time.

GFP-HAS3 Protein Turnover Time in REKs—Cycloheximide caused ~50% reduction in hyaluronan synthesis in 4 h, a result in line with reports on cultured chondrocytes, mesothelioma cells, and orbital fibroblasts (23–26). Although the reduction of hyaluronan synthesis can be due to protein targets of cycloheximide other than HAS, the present microscopic observations indicating a 4–5 h turnover time of GFP-HAS3 were completely in line with a 2–3 h-half-life of HAS3 protein and the observed decline in hyaluronan synthesis.

During the cycloheximide block, microscopic examination suggested that plasma membrane was continuously supplied with new GFP-HAS until the stores in the Golgi area were exhausted. The presence of a latent form of endogenous HAS was suggested by experiments showing that protein kinase C activation stimulates hyaluronan synthesis partly through mechanisms independent of protein synthesis (30, 31). Regulated mobilization of HAS from the Golgi complex would provide cells a way of responding to challenges that require rapid activation of hyaluronan synthesis. Indeed, following introduction of epidermal growth factor, hyaluronan synthesis of REKs is more than tripled in 3 h (15), which supports the idea of mechanisms acting before transcription, translation, and post-translational processing bringing the Has gene activation effect to the plasma membrane. The transfer from ER to the Golgi complex alone took about 1 h, as suggested by the data from the experiments from brefeldin A recovery. Possible regulation of hyaluronan synthesis through gating of the HAS traffic from the Golgi complex to the plasma membrane can be studied in the future by direct microscopy of live cells. The present data thus suggested that the relatively slow inhibition by cycloheximide of hyaluronan synthesis is due to the continued flow of latent HAS from intracellular compartments to the plasma membrane for activation.

GFP-HAS Plasma Membrane Dwell Time and Endocytosis—Treatment of REKs for 2 h with brefeldin A almost totally depleted the plasma membrane from GFP-HASs, suggesting that their turnover time on the plasma membrane is 2 h or less. This time was consistent with the 4–5 h total turnover time of the GFP-HAS3, suggested by the cycloheximide experiments, and also with the steady state distribution of the GFP signal in the different compartments of REKs. Assuming a chain elongation rate of three monosaccharides/s (32), ~3 h would be required for the synthesis of a 6 × 10^6 Da hyaluronan polymer, the size found in REKs (33). Based on these estimates, each HAS protein synthesizes only one hyaluronan chain in REKs, as also suggested by Ref. 34, before it is recycled.

From the plasma membrane, GFP-HAS cycled back in endosomes, presumably on its way to the lysosomes for degradation. The nature of signal for HAS internalization is obscure. It is, however, easy to imagine that the cessation of hyaluronan synthesis and the eventual detachment of the huge hyaluronan chain leave the HAS more susceptible to endocytosis. This, and the question of whether the endocytosed GFP-HAS is intact and could be enzymatically active if recycled back to the cell surface, are new problems to be addressed in the future.

HAS Insertion Sites on Plasma Membrane—The images of GFP-HAS2 and GFP-HAS3 in REKs suggested that the signal was not evenly distributed over the plasma membrane but rather as discrete spots. The same finding was observed when GFP-xHas1 was transfected into oocytes (14). REKs are also known to display cell surface hyaluronan as patches, 100–200 nm cell, perhaps corresponding to the HAS sites (35). The present data, a part of which was obtained from high resolution three-dimensional reconstructions of live cells, provided important novel information on this issue, suggesting that these GFP-HAS enrichment sites were actually cell surface protrusions. The targeting mechanism and the importance of this location are under further investigation.

Hyaluronan Synthesis Start Site and Control of HAS Activation—Colocalization analysis of GFP-HAS and hyaluronan allowed us for the first time to address the question of whether hyaluronan chains associate with HAS when still in the ER, Golgi complex, or post-Golgi vesicles. The conclusion is that intracellular hyaluronan and HAS only occur together in vesicles derived from their co-endocytosis from plasma membrane. Therefore, if HAS is activated intracellularly in REKs before entering plasma membrane, the kinetics of this phase must be very fast.

The depletion of plasma membrane GFP-HAS3 following treatment with the hyaluronan synthesis inhibitor 4-MU was somewhat surprising, considering the fact that its major effect is thought to be through cellular UDP-GlcUA starvation, with no direct influence on the enzyme itself (36). However, the finding is consistent with an idea that HAS entrance to, or retention on, the plasma membrane depends on ongoing hyaluronan synthesis. This suggestion is also in line with the finding that the enzymatically inactive D216A mutant was present in the same intracellular compartments as the wild type HAS3 but not on the plasma membrane.

What are the intrinsic factors in HAS, or extrinsic conditions in the environment, that allow hyaluronan synthesis specifically on the cell surface? This key question becomes even more acute by the present demonstration that there is a relatively large, latent pool of intracellular HAS. Although the current data did not offer a direct solution, we propose a new hypothesis whereby enzymatic activation drives HAS to the plasma membrane, and the continuing synthetic activity maintains it at this location.

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