Hyaluronan synthesis induces microvillus-like cell surface protrusions

Anne Kultti1, Kirsi Rilla2, Riikka Tiihonen2, Andrew P. Spicer3, Raija H. Tammi2, and Markku I. Tammi4

From the 4Department of Anatomy, University of Kuopio, FIN-70211 Kuopio, Finland and 5Texas A&M University System Health Science Center, Institute of Biosciences and Technology, Houston, Texas 77030

Hyaluronan synthases (HASs) are plasma membrane enzymes that simultaneously elongate, bind, and extrude the growing hyaluronan chain directly into extracellular space. In cells transfected with green fluorescent protein (GFP)-tagged Has3, the dorsal surface was decorated by up to 150 slender, 3–20-μm-long microvillus-type plasma membrane protrusions, which also contained filamentous actin, the hyaluronan receptor CD44, and lipid raft microdomains. Enzymatic activity of HAS was required for the growth of the microvilli, which were not present in cells transfected with other GFP proteins or inactive GFP-Has3 mutants or in cells incubated with exogenous soluble hyaluronan. The microvilli induced by HAS3 were gradually withered by introduction of an inhibitor of hyaluronan synthesis and rapidly retracted by hyaluronidase digestion, whereas they were not affected by competition with hyaluronan oligosaccharides and disruption of the CD44 gene, suggesting independence of hyaluronan receptors. The data bring out the novel concept that the glyocalyx created by dense arrays of hyaluronan chains, tethered to HAS during biosynthesis, can induce and maintain prominent microvilli.

Vertebrate cells display slender plasma membrane protrusions like filopodia, microspikes, and microvilli, the formation and maintenance of which are thought to depend on the dynamics of a bundle of actin filaments in the core of these extensions (1). In selected environments, most of the components in the actin polymerization apparatus, when overexpressed or experimentally activated, have been reported to increase the growth of filopodia or microvilli (2–7). This suggests that there is a strong intrinsic potential in cells to display these extensions, ready for implementation by factors even outside the effector and signaling chains directly related to actin. Indeed, at the molecular level, mechanisms that induce the growth of microvilli in vivo are still somewhat obscure (8). We present a novel factor, active hyaluronan synthesis, apparently unrelated to any of the previously described signaling or actin polymerization processes, that triggers extensive microvillus formation in several cell types. Interestingly, the process is primarily driven by the cell surface glyocalyx rather than intracellular systems.

Hyaluronan is an ubiquitous pericellular and extracellular matrix glycosaminoglycan important in embryonic development (9), wound healing (10), inflammation (11, 12), mammalian fertilization (13), and cancer (14). It is involved in cell adhesion (15), migration (16–19), proliferation and epithelial-mesenchymal transition (9), resistance to apoptosis, and multidrug resistance (14). Some of its effects are thought to be mediated by an ability to form a hydrated pericellular matrix, a coat that can modify cellular interactions with other matrix components and neighboring cells, directly regulating processes such as differentiation and migration (20). Some of the biological signals elicited by hyaluronan are dependent on its CD44 receptor on cell surfaces (21), whereas some of the signals have not been directly connected to any receptor or interacting molecule (9).

Hyaluronan synthases (HAS1, HAS2, HAS3)3 are a family of vertebrate plasma membrane enzymes synthesizing hyaluronan. They are integral membrane proteins that catalyze the alternate addition of glucuronic acid and N-acetylglucosamine from their UDP derivatives to a growing hyaluronan polymer, continuously translocated through the plasma membrane into extracellular matrix (22) and eventually reaching a molecular mass up to 107 Da (23). There are distant homologues of the vertebrate HAS also in Streptococcus and Pasteurella bacteria (24).

All vertebrate HAS isoenzymes are apparently synthesized into the membrane of the endoplasmic reticulum and transported through Golgi apparatus to the plasma membrane (25, 26). To be enzymatically active, HASs are not known to bind any other protein (27). However, Streptococcus HAS binds several cardiolipin molecules to reach maximal activity (28), but no such essential lipid has been found for vertebrate HAS (29). Despite the apparently missing protein or lipid interactions that could aid in vertebrate HAS targeting to a specific membrane domain, Xenopus green fluorescent protein Has1 fusion protein (GFP-Has1) translocated into oocytes accumulates at some uncharacterized sites on the plasma membrane (25). In our recent study on GFP-labeled murine Has2 and Has3 transacted into epidermal keratinocytes, it turned out that the plasma membrane domains particularly enriched in HAS were cell surface protrusions (26).

In this study, we show that HAS not only seeks its way to existing cell surface protrusions but actually creates specific microvilli up to 20 μm tall on several cell types. The induction and maintenance of these microvilli are completely dependent on an ongoing synthesis of hyaluronan by HAS, most concentrated in these structures.

EXPERIMENTAL PROCEDURES

Cell Culture—The human breast adenocarcinoma cell line, MCF-7, was cultured in minimum essential medium (Invitrogen, Paisley, Scot-

3 The abbreviations used are: HAS, hyaluronan synthase; bHABC, biotinylated hyaluronan binding complex; FBS, fetal bovine serum; GFP, green fluorescent protein; SEM, scanning electron microscopy, TEM, transmission electron microscopy; RHAMM, receptor for hyaluronan-mediated motility; MDCK, Madin-Darby canine kidney cells; TRU, turbidity-reducing units.
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land, UK) supplemented with 5% fetal bovine serum (FBS, PAA Laboratories GmbH, Pasching, Austria), 2 mM glutamine (Sigma) and 50 µg/ml streptomycin sulfate and 50 units/ml penicillin (Sigma). Cells were passaged twice a week at a 1:5 split ratio using 0.05% trypsin (w/v) in 0.02% EDTA (w/v) (Biochrom, Berlin, Germany). The SKOV-3 human ovarian adenocarcinoma cells (ATCC, Manassas, VA) were maintained in McCoy’s 5A medium with 10% FBS (Euroclone, Milan, Italy), and glutamine and antibiotics as above. Madin-Darby canine kidney epithelial (MDCK) cells (from Dr. Donald MacCallum, Ann Arbor, MI) were cultured in Dulbecco’s modified Eagle’s medium high glucose (Euroclone) supplemented with 10% FBS, glutamine, and the antibiotics. Rat epidermal keratinocytes were cultured in minimum essential medium (Euroclone) supplemented with 5% FBS, glutamine, and the antibiotics.

Primary mouse epidermal keratinocytes were isolated and cultured as described by Hager et al. (30). Skin was obtained from newborn wild type (C57Bl/6) and CD44−/− mice (obtained from Dr. Paul Noble, West Haven, CT and originally developed by Dr. Tak Mak, Toronto, Canada). The Animal Care and Use Committee of the University of Kuopio approved the study protocol. Epidermis and dermis were separated with Dispase (type II, Roche Applied Science, Mannheim, Germany), and the cells were released from the tissues with a brief treatment of 0.05% trypsin in 0.02%-EDTA. The keratinocytes were suspended in medium and plated at 50,000 cells/well on 8-well chambered cover glass slides (Nalge Nunc, Naperville, IL) coated with 1 µg/cm² of type IV collagen (BD Biosciences). This medium contains Eagle’s minimum essential medium (BioWhittaker, no calcium) supplemented with 0.06 mM Ca²⁺, 8% FBS (HyClone, Logan, UT) treated with Chelex (Bio-Rad), 0.4 µg/ml hydrocortisone (hydrocortisone hemisuccinate, Sigma), 0.75 mM aminoguanidine nitrate (Aldrich, Steinheim, Germany), 2 ng/ml epidermal growth factor (Sigma), 10⁻¹⁰ M choleratoxin (Sigma), and 3T3-fibroblast conditioned medium (1:1).

HaCat cells were a kind gift from Dr. Norbert Fusenig (Heidelberg, Germany). They were cultured in Dulbecco’s modified Eagle’s medium (high glucose, Invitrogen, Paisley, UK) supplemented with 10% FBS (PAA Laboratories). For passaging, the cells were treated with EDTA (0.02% in Dulbecco’s phosphate-buffered saline, Sigma) for 5 min and then with 0.05% trypsin in 0.02% EDTA (Biochrom) for 10 min. The cells were maintained by plating at a 1:10 split ratio twice a week.

Transfections—Subconfluent cell cultures were transfected using FuGENE 6 reagent (Roche Applied Science). Transfected constructs were mouse Has3 cDNA in-frame with an N-terminal GFP fusion protein in the pcIneo vector (31), GFP alone in the pcIneo vector (31), GFP-Has3 pcIneo with a D216A targeted mutation (31), and human taurine transporter (Hannu Karjalainen, University of Kuopio, Department of Anatomy) in EGFP-N1 vector (Molecular Probes, Eugene, OR). The cells were examined 16–48 h after transfection. Culture medium was collected and subjected for hyaluronan enzyme-linked sorbent assay 48 h after transfection.

Treatments of the Cultures—0.2 mg/ml HA₅ or HA₁₀ oligosaccharides (Seikagaku Kogyo Co., Tokyo, Japan) were added to live MCF-7 cells after or during transfection. Streptomycyes hyaluronidase (2.5–10 TRU/ml, Seikagaku Kogyo Co.), 4-methylumbelliferone (1 mM, Sigma), or brefeldin A (5 µg/ml, Sigma) were added to living cell cultures a day after transfection. Non-transfected MCF-7 cells were treated with high molecular mass hyaluronan (AmVisc™ plus, Chiron Vision, Lyon, France) overnight, fixed, and stained for lipid raft microdomains as described below. Microtubules in MCF-7 cells overexpressing HAS3 were removed by 30 min of incubation on ice followed by nocodazole (63 mM, Sigma) for 30 min at 37°C. GFP-Has3-transfected MCF-7 cells were treated with 5 µg/ml latrunculin B (Calbiochem, La Jolla, CA) for 2 h at 37°C, fixed, and stained with phalloidin as described below. Hermes 1 (a partly blocking antibody for CD44, 5 µg/ml), was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City) (32). The R36 antibody (blocking antibody for receptor for hyaluronan-mediated motility (RHAMM), 20 µg/ml) was a gift from Dr. Rashmin Savani, University of Pennsylvania School of Medicine, Philadelphia, PA. Cholesterol was depleted in the transfected MCF-7 cells by treating the cells with 1% methyl-β-cyclodextrin (Sigma) for 30 min-2 h.

Assay of Hyaluronan—A subconfluent MCF-7 cell culture on 24-well plates was transfected with GFP-Has3, changed next day to a fresh medium (with 5% FBS), and cultured for 24 h before counting the cells and harvesting the medium for the enzyme-linked sorbent assay of hyaluronan as described previously (33).

Red Blood Cell Exclusion Assay—Fixed sheep red blood cells (Sigma) were suspended in phosphate-buffered saline containing 0.1% bovine serum albumin, washed, and suspended in phosphate-buffered saline. Aliquots of the suspension were added to a cell culture and allowed to settle for at least 15 min at 37°C before microscopy.

Localization of Hyaluronan—For the light microscopic detection of hyaluronan, cells were seeded on collagen type I precoated 8-well chambered cover glasses and transfected as described above. The slides were fixed with 2% paraformaldehyde, washed, permeabilized, and probed with 2 µg/ml biotinylated hyaluronan binding complex (bHABC) as described previously (26).

For transmission electron microscopy (TEM), the GFP-transfected cells were fixed and stained for hyaluronan as described previously (34). The cells were permeabilized using 0.05% saponin in all incubation buffers and incubated overnight at 4°C with 10 µg/ml bHABC followed by 2 h with streptavidin peroxidase and 20 min with 0.03% 3,3′-diaminobenzidine (Sigma) containing 0.03% H₂O₂. After postfixation with reduced osmium tetroxide, the cells were dehydrated and embedded in Spurr’s resin. Thin sections were stained with uranyl acetate and viewed using a JEOL 1200 EX microscope.

Scanning Electron Microscopy (SEM)—For SEM, cells were grown and transfected on 13-mm coverslips. After 48 h, the cultures were washed and fixed with 4% paraformaldehyde for 2 h at room temperature and dehydrated through a graded series of ethanol. After critical point drying, cells were shadowed with gold and photographed on an XL30 TMP environmental SEM (FEI Company, the Netherlands) at 15 kV.

Immunofluorescence Staining—For the staining of CD44, actin cytoskeleton, microtubules, and lipid rafts, transfected MCF-7 cells were fixed, permeabilized, and blocked as described above and incubated overnight at 4°C with anti-CD44 monoclonal antibody (Hermes 3, 1:100, a generous gift from Dr. Sirpa Jalkanen, Turku, Finland) or anti-β-tubulin (1:500, Roche Applied Science) in 1% bovine serum albumin. After washing, the cells were incubated for 1 h with Texas Red conjugated secondary antibody (Vector, 1:500). For the visualization of the actin filaments, the cells were incubated with Alexa Fluor® 594 phallolidin (5 units/ml, Molecular Probes) for 1 h. Lipid raft microdomains at plasma membrane were visualized by a Vybrant® lipid raft labeling kit (Molecular Probes).

Confocal Imaging—The micrographs were obtained with an Ultraview® confocal scanner (PerkinElmer Life Sciences, Wallac-LSR, Oxford, UK) on a Nikon Eclipse TE300 microscope with a 60× NA 1.4 and 100× NA 1.4.
The maximum length of the microvilli was (Fig. 1) protrusions were not present in MCF-7 cells transfected with GFP only with a plasmid containing only GFP (Fig. 1). Ronan (Fig. 1). Erythrocytes are excluded from a pericellular space occupied by hyaluronan. Transfection efficiency was 20–50%, based on the estimation of brightly transfected cells/24 h, about 100-fold increase as compared with control cultures parent MCF-7 cells, whereas GFP-Has3 transfection increased the synthesis in MCF-7 Cells—Since hyaluronan has been shown to enhance potentially malignant cell properties, such as epithelial-mesenchymal transition, resistance to apoptosis, and multidrug resistance (14), we started transient transfections of Has3 coupled to GFP into MCF-7, a relatively non-aggressive human breast cancer cell line, to investigate the effects of increased hyaluronan synthesis on the phenotype of these cells (35). Hyaluronan synthesis was relatively low in the parent MCF-7 cells, whereas GFP-Has3 transfection increased the secretion of hyaluronan into culture medium up to 200 ng/10,000 cells/24 h, about 100-fold increase as compared with control cultures transfected with a vector containing a GFP protein alone (Table 1). The transfection efficiency was 20–50%, based on the estimation of brightly fluorescent cells 1 day after transfection.

The cell-associated hyaluronan was also increased in GFP-Has3-transfected cells as indicated by the classical assay in which sedimented erythrocytes are excluded from a pericellular space occupied by hyaluronan (Fig. 1b). An exclusion space did not exist around cells transfected with a plasmid containing only GFP (Fig. 1a).

The Hyaluronan Coat Contains GFP-HAS3-positive Cell Protrusions—Careful examination of the erythrocyte sedimentation assays revealed that the “empty” space representing the hyaluronan coat actually contained GFP-HAS3-positive cell protrusions (Fig. 1b). Similar villous protrusions were not present in MCF-7 cells transfected with GFP only (Fig. 1a). Hyaluronan was also increased, and microvilli were induced on cells transfected with a plasmid containing GFP-Has2 (data not shown). The maximum length of the microvilli was ~20 μm, and the number of those longer than ~3 μm ranged from a few to about 150/cell. Fixation of the cell cultures with aldehydes usually resulted in a considerable truncation of the microvilli and partial loss of the hyaluronan coat. However, even after fixation, standard staining with the bHABC and visualization by fluorescent streptavidin, microvilli, and a definite layer of hyaluronan were recognized on cells transfected with GFP-Has3 (Fig. 1d) but not on GFP-only cells (Fig. 1c). There was also a strong signal from intracellular GFP-Has3, representing its traffic in the secretory pathway through the endoplasmic reticulum, Golgi complex, and endocytic vesicles (26).

The ultrastructure of the microvilli was studied in GFP-Has3-transfected MCF-7 cells stained for hyaluronan with the bHABC probe, visualized by 3,3′-diaminobenzidine precipitates, and observed by TEM (Fig. 1e). As in the confocal images, the transfected cells were associated with hyaluronan, visualized as the knob- and ribbon-like deposits on the microvilli (Fig. 1e). The TEM analysis showed that the microvilli were slender; their average diameter was ~120 nm. As a consequence, the odds were very low that any longer stretch of microvillus would occur in the ~70-nm-thick plane of the electron microscopy section. A more comprehensive view was obtained with SEM that revealed ~130-nm-diameter microvilli (Fig. 1f). The microvilli were collapsed onto the cells, obviously due to the dehydration required for the visualization by gold sputtering on the cells. Cells (Fig. 1f, asterisk) and cultures not transfected with GFP-Has3 showed no such microvilli.

### Table 1

Hyaluronan concentration in the culture media of MCF-7 cells transfected with GFP and GFP-Has3

| Transfection               | Hyaluronan production over 24 h ng/10,000 cells | Fold increase |
|----------------------------|-----------------------------------------------|---------------|
| No transfection            | 1.8 ± 0.4                                     | 1.0 ± 0.0     |
| GFP transfection           | 2.6 ± 0.4                                     | 1.6 ± 0.3     |
| GFP-Has3 transfection      | 193.5 ± 55.2                                  | 122.0 ± 43.7  |

Means ± S.E. of three independent experiments are shown.

### Results

GFP-Has3 Transfection Causes a Major Induction of Hyaluronan Synthesis in MCF-7 Cells—Since hyaluronan has been shown to enhance potentially malignant cell properties, such as epithelial-mesenchymal transition, resistance to apoptosis, and multidrug resistance (14), we started transient transfections of Has3 coupled to GFP into MCF-7, a relatively non-aggressive human breast cancer cell line, to investigate the effects of increased hyaluronan synthesis on the phenotype of these cells (35). Hyaluronan synthesis was relatively low in the parent MCF-7 cells, whereas GFP-Has3 transfection increased the concentration of the cell cultures with aldehydes usually resulted in a considerable truncation of the microvilli and partial loss of the hyaluronan coat. However, even after fixation, standard staining with the bHABC and visualization by fluorescent streptavidin, microvilli, and a definite layer of hyaluronan were recognized on cells transfected with GFP-Has3 (Fig. 1d) but not on GFP-only cells (Fig. 1c). There was also a strong signal from intracellular GFP-Has3, representing its traffic in the secretory pathway through the endoplasmic reticulum, Golgi complex, and endocytic vesicles (26).

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The Microvilli Grow into Their Full Length in a Few Hours and Are Relatively Stable—To evaluate the growth rate of the microvilli, transfected cells were first treated with brefeldin A, which blocks the traffic of GFP-HAS3 from the Golgi to the plasma membrane and returns the protein to the endoplasmic reticulum (26). Time-lapse imaging of individual cells following removal of brefeldin A indicated that the first small microvilli emerged after 1.5–2 h (Fig. 2, a and b), corresponding to the time required for GFP-HAS3 to travel from the endoplasmic reticulum to the plasma membrane (26). The number and height of the microvilli gradually increased up to 4–5 h after removal of the brefeldin A block and then remained at that state (Fig. 2). Individual microvilli were relatively stable as suggested by time-lapse imaging for shorter, 5–15-min periods (Supplemental Movie 1).

The Microvilli Arise in Several Cell Types Transfected with GFP-Has—The expression of GFP-Has3 resulted in a “hedgehog” appearance in all epithelial cell types studied so far, including SKOV-3 ovarian cancer cells (Fig. 3, a and b), MDCK kidney epithelial cells (Fig. 3, c and d) and rat epidermal keratinocytes, human (HaCaT), and mouse primary keratinocytes, suggesting that their induction by GFP-HAS3 is ubiquitous. The appearance rate of the microvilli was somewhat slower in MDCK cells as compared with the ovarian or breast cancer cell lines, but 48 h after transfections, these protrusions were present in all these cell types.

The GFP-HAS3 Induced Microvilli Are Supported by Actin Filaments—Even in the partially truncated structures resulting from fixation and staining with phalloidin, it was obvious that filamentous actin was present in the core of the microvilli (Fig. 4, b and c), whereas they showed no signal with an antibody against ß-tubulin (data not shown). Accordingly, treatment with latrunculin B to degrade actin filaments destroyed the microvilli (Fig. 4, d) and eventually detached the cells. In contrast, nocodazole that disrupts microtubules had no effect on microvilli or cell morphology (data not shown). These results indicate that the microvilli largely rested on actin filaments.

Immunostaining for lipid raft microdomains utilizing cholera toxin subunit B showed their widespread distribution on MCF-7 cells, but the signal was particularly prominent in the microvilli (Fig. 5, b). There was a partial colocalization between GFP-HAS3 (Fig. 5, a) and the lipid raft staining, both in the microvilli and in the cell body (Fig. 5, c). Introduction of methyl-ß-cyclodextrin, an agent that abducts cholesterol from the cell, resulted in the disappearance of the microvilli (Fig. 5, d). These data suggest that cholesterol, probably associated with the lipid raft material, is vital for the maintenance of the microvilli.

The Microvilli Are Created and Maintained by Active Hyaluronan Synthesis—To distinguish between the alternatives that the microvillus formation was triggered specifically by the GFP-HAS3 protein or any
GFP-tagged membrane protein, we transfected into MCF-7 cells a GFP-labeled taurine transporter, which is also inserted into the plasma membrane. The GFP-taurine transporter appeared to label all plasma membranes, including cell protrusions (Fig. 6, c and d). However, the protrusions visualized by taurine transporter were often branched, were variable in thickness, and were lying on the lateral aspect of the cell, (Fig. 6, c and d), whereas the HAS-dependent microvilli were unbranched, mostly projected up from the dorsal side of the cell, and typically contained a knob at the tip (Fig. 6, a and b).

To reveal whether the HAS protein or the hyaluronan synthesized by HAS was responsible for the induction of the microvilli, a missense (D216A) mutation was introduced into the active center of GFP-HAS3. No microvilli were found in cells transfected with this enzymatically inactive mutant (Fig. 6, e and f). Likewise, enzymatically inactive GFP-HAS3 proteins with short C-terminal truncations did not support microvillus formation (data not shown).

Depletion of the UDP-glucuronic acid precursor pool by 4-methylumbelliflorone severely inhibits hyaluronan synthesis in some cell types (26, 36), including MCF-7 cells (data not shown). The microvilli were mostly retracted 4 h after introduction of 4-methylumbelliflorone (Fig. 7, a and b), a further indication that active hyaluronan synthesis was required for their maintenance.

The Microvilli Are Not Dependent on CD44—Most of the microvilli were strongly positive for the hyaluronan receptor, CD44 (Fig. 7c). Since the influences of hyaluronan on cells are often mediated by CD44, we added soluble, high molecular mass hyaluronan in various concentrations up to 1 mg/ml to non-transfected cultures to stimulate the signaling through CD44. No induction of microvilli tall enough for confocal detection was found with any concentration of exogenous hyaluronan, as monitored by lipid raft staining. Likewise, cultures incubated with a blocking antibody against CD44 did not disrupt the microvilli created by GFP-HAS3. Incubations with high concentrations of hyaluronan decasaccharides, displacing hyaluronan from CD44, were also without effect when introduced after the microvilli had formed or at the time of transfection (Fig. 7, f and g). To further check on this somewhat unexpected result, we expressed GFP-HAS3 in epidermal keratinocytes derived from CD44 knock-out mice. Since the same types of microvilli were found on wild type and CD44−/− cells (Fig. 7, d and e), we conclude that CD44 is not necessary for their formation.

To probe for the involvement of receptors other than CD44, we incubated the GFP-HAS3-transfected cultures with blocking antibodies against RHAMM, resulting in no change in the microvilli and suggesting that RHAMM is not involved either (data not shown). Microarray data on MCF-7 cells suggested that these cells do not express significant levels of the hyaluronan receptors LYVE-1, layilin, and stabilin 2 (hyaluronan receptor for endocytosis) (data not shown). The microvilli were also resistant to hyaluronan decasaccharides that should compete hyaluronan off from all currently known hyaluronan-binding proteins.

FIGURE 4. Localization of filamentous actin in the microvilli of MCF-7 cells transfected with GFP-Has3. A day after GFP-Has3 transfection, the cells were fixed and stained with Alexa Fluor® 594-phalloidin. Vertical sections obtained from stacks of horizontal confocal images are shown. Actin (red) is localized at the core of the GFP-Has3 (green) positive microvilli (a–c). To disrupt the actin cytoskeleton, GFP-Has3-transfected cells were treated with latrunculin B, resulting in the disappearance of the microvilli (d). Bars: 10 μm.

FIGURE 5. Localization of lipid rafts in MCF-7 cells transfected with GFP-Has3. The GFP-Has3-transfected cells were fixed, and lipid rafts were detected with cholera toxin subunit B (red). A vertical section of a cell expressing GFP-Has3 is shown in a, the same cell stained for lipid rafts is shown in b, and the merged images are shown in c. Merged images of GFP-Has3 and lipid rafts after a 30-min methyl-β-cyclodextrin treatment are shown in d, demonstrating the disappearance of the microvilli. Bar: 10 μm.

FIGURE 6. GFP-HAS3 specificity in the microvillus induction. The GFP-signal of live MCF-7 cells are shown 20 h after transfection with GFP-Has3 (a and b), a plasma membrane taurine transporter (c and d), and GFP-Has3 with an inactivating missense mutation (e and f). Compressed image stacks of horizontal optical sections (a, c, and e) and vertical sections (b, d, and f) are shown. Note the absence of dorsal microvilli in c–f. Bar: 10 μm.

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Hyaluronan-induced Microvilli

FIGURE 7. Effect of hyaluronan synthesis inhibition, hyaluronan receptor elimination, and hyaluronidase digestion on microvilli induced by GFP-HAS3. A suspension of fixed red blood cells was allowed to sediment on GFP-HAS3-positive MCF-7 cells and viewed before (a) and after (b) a 4-h treatment with the hyaluronan synthesis inhibitor 4-methylumbelliferone to demonstrate the shrinkage of the hyaluronan coat and microvilli. CD44 is localized on the microvilli of GFP-Has3-transfected cells, as detected by anti-CD44 monoclonal antibody (c). Primary keratinocytes from a wild type mouse (d) and one deficient for CD44 (e) show similar microvilli following GFP-Has3 transfection. The addition of 200 μg/ml hyaluronan decasaccharides in the growth medium of MCF-7 cells at the time of transfection does not prevent microvilli formation (f) as compared with control cultures (i). Treatment of a GFP-HAS3-positive MCF-7 cell (h) with 10 TRU/ml Streptomyces hyaluronidase removes the microvilli in 1 min (j). Single optical sections are shown in (a, b, h, and i), compressed images of horizontal stacks in (f and g) and vertical sections in (c, d, and e). See Supplemental Movie 2 for the retraction of the microvilli by hyaluronidase treatment. Bars: 10 μm.

(Data not shown). Indeed, these studies provided no data to suggest that cell surface receptors for hyaluronan are required for the protrusions.

Degradation of Cell Surface Hyaluronan Destroys the Microvilli—The experiments above indicated that active hyaluronan synthesis is required for the creation and maintenance of the microvilli but did not make a distinction between the importance of the transferase activity as such and the hyaluronan chains undergoing synthesis and still attached to HAS. This issue was studied by introduction of Streptomyces hyaluronidase, an endo-enzyme completely specific for the degradation of hyaluronan. In live cell cultures, the addition of Streptomyces hyaluronidase to a final concentration of 2.5–10 TRU/ml detached and degraded cell surface hyaluronan. This caused disruption of the microvilli in a few minutes (Fig. 7, h and i, and Supplemental Movie 2). The same occurred in cultures treated with testicular and Streptococcus hyaluronidases. The microvilli are thus dependent on the cell surface hyaluronan, retained there by HAS.

DISCUSSION

The present data establish that hyaluronan synthesis, by elevated HAS enzyme activity, creates morphologically distinct, up to 20-μm-long microvilli on several types of cultured cells. The finding that a cell surface glycosaminoglycan caused such a major modification in the form adopted by the plasma membrane was quite unexpected. Indeed, the microvilli triggered by HAS expression were entirely dependent on the high molecular mass hyaluronan chains on the cell exterior. This is obvious since they were absent in cells overexpressing an unrelated plasma membrane protein or an inactive HAS mutant. Further, they gradually withered in the presence of an inhibitor of hyaluronan synthesis and immediately retracted after introduction of hyaluronidases, which degrade the hyaluronan chains.

A high expression level of HAS was required to induce microvilli that were long enough for easy detection by confocal microscopy. In the MCF-7 cells expressing GFP-HAS, hyaluronan synthesis was ~30 pg/cell/12 h. For most cell types in culture, the rates of stimulated hyaluronan synthesis range between 2 and 15 pg/cell/12 h (37–40), but quantities as high as 30 and 106 pg/cell/12 h have been reported in foreskin fibroblasts and mesothelial cells, respectively (41, 42). Hyaluronan synthesis rates equal or higher than that after Has3 transfections are thus possible through endogenous HAS expression. It is also likely that hyaluronan synthesis rates lower than that in the present study induce similar but less extended microvilli. Indeed, transmission electron microscopy revealed increased numbers of protrusions of similar diameter as in the present study, which were induced by stimulation of hyaluronan synthesis in keratinocytes by fibroblast growth factor 7 (FGF-7) (33). Since the microvilli are fragile and beyond the resolution of light microscopy without fluorescent markers, it is currently not known how often they are present in cells with endogenously elevated hyaluronan synthesis.

It was also unexpected that the main cell surface hyaluronan receptor CD44, and probably other receptors activated by high molecular mass hyaluronan, were not necessary for the growth of the microvilli. How does pericellular hyaluronan signal for microvillus formation if not through binding to cell surface receptors? The remaining two alternatives are either that the HAS protein itself, when inserted in the plasma membrane, induces a signal for microvillus formation or that the large hyaluronan chains tethered to plasma membrane change the membrane behavior without specific binding to membrane proteins. The former alternative seems less likely since no interactive proteins have been found for HAS, and neither is there a dominant negative effect on hyaluronan synthesis following transfection with point-mutated Has-3 (26). The latter alternative, that just hyaluronan tethering to plasma membrane triggers the signal, perhaps by creating forces that deform the membrane (43) due to crowding of adjacent, large solution domains of hyaluronan molecules, is more attractive. This idea would also allow plasma membrane “stretching” effects by exogenous hyaluronan to support microvilli in those cells or conditions in which CD44 is highly active. Interestingly, hyaluronidase treatment is almost as effective as latrunculin A in reducing the mechanical strength of thin plasma membrane protrusions, suggesting that hyaluronan can also act like an extra-
cellular cytoskeleton, organizing (44) and supporting the membrane (45).

Bursts of intracellular hydrostatic pressure can detach plasma membrane from cortical cytoskeleton and initiate local plasma membrane protrusions, which are also modified by extracellular osmotic pressure and cross-linking of cell surface glycoproteins (46). The physicochemical forces created by the high concentration of membrane-tethered hyaluronan could also influence membrane kinetics. It can be envisioned that spots of plasma membrane with weak cortical cytoskeleton attachment are preferential fusion sites for HAS-containing vesicles arriving from Golgi, and at the same time, prone to bulging by the swelling pressure caused by hyaluronan that starts to grow on the external side of the membrane.

Positive and negative membrane curvatures occur in the basis and stem parts of the microvilli, respectively (47). Such acute membrane bends accommodate special lipids, often with wedge-shaped membrane insertions or bulky head groups, and are coupled to scaffolding proteins that provide the force to bend the membrane (48). Thus, HAS may be accompanied by such special lipids, and the hyaluronan attached to HAS would form the scaffolding force to promote and stabilize the highly curved microvilli membrane.

The hyaluronan-induced membrane deformation can also be considered in terms of water flow. The rapid hydration of newly synthesized cell surface hyaluronan may draw water through membrane water channels, or a pore formed by HAS itself, shrinking the surrounding cytoplasm toward a microvillous form.

Overexpression of the cell surface heparan sulfate proteoglycan syndecan 3 induces slender cell surface protrusions through a mechanism that is difficult to couple to any previously known cytoplasmic signaling system (49). Likewise, transfection of PA2.26 antigen, a plasma membrane glycoprotein with mucin type oligosaccharides, creates filopodia and ruffles (50). Furthermore, high expression of a cadherin homologue without an intracellular domain to bind cytoskeleton still increases microvillus length (8). Cell surface glycoconjugates or bulky membrane molecules other than hyaluronan may thus also have these control and support functions on plasma membrane protrusions.

A number of questions, especially concerning the role of actin filaments, arise from the present findings. Is any component of the actin cytoskeleton interacting directly or indirectly with HAS? Are there determinants in the cortical cytoskeleton that serve as specific initiation sites for the often limited number of microvilli on each cell? Do actin filament bundles have an active role in microvillus growth, or are they passively filling the stem of the growing microvillus, as described previously for another type of membrane protrusion (46)?

Microvillus-type cell protrusions represent a distinct cellular compartment that harbors important regulatory functions (43). Among others, multidrug resistance transporter proteins (51, 52) and ErbB2 receptors (53) reside in microvilli. Hyaluronan synthesis increases ErbB2 activation (9), and ErbB2 stimulates hyaluronan synthesis via a phosphoinositide 3-kinase-dependent positive feedback loop, resulting in enhanced expression and activity of multidrug resistance protein 1 (54). Enhanced hyaluronan synthesis thus creates microvilli for its own synthesis, and the same hyaluronan-induced microvilli can offer a preferred site for both ErbB2 signaling and multidrug resistance proteins. The microvillar platform induced by hyaluronan seems highly interesting in view of the recently established role of hyaluronan as a promoter of malignant cellular phenotype (14) and an unfavorable factor for patient survival in several epithelial cancers (55–57). In addition, cell surface protrusions make contacts to extracellular matrix and other cells, sense and interact with the environment, and lead to new attachment sites and cell migration (1). Hyaluronan synthesis increases the migration rate of different cell types (17–19), but it is currently not known which type of protrusions are involved. The present finding that hyaluronan tethered to plasma membrane strongly promotes cell surface protrusions offers a novel view to a number of previous reports indicating that hyaluronan stimulates cellular locomotion, multidrug resistance, ErbB2 signaling, and other poorly understood impacts of hyaluronan in cellular behavior.

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