Extracellular UDP-Glucose activates P2Y$_{14}$ receptor, induces signal transducer and activator of transcription 3 (STAT3) T705 phosphorylation and binding to hyaluronan synthase 2 ($\text{HAS2}$) promoter, stimulating hyaluronan synthesis of keratinocytes

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Running title: Extracellular UDP-Glucose regulates HAS2 expression

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Capsule

Background: The secretion and possible functions of extracellular UDP-sugars in epidermal keratinocytes are not known.

Results: UDP-glucose activates P2Y$_{14}$ receptor and JAK2, increases STAT3 tyr705 phosphorylation, and enhances transcription of hyaluronan synthase 2 ($\text{HAS2}$).

Conclusion: UDP-glucose release signals for enhanced $\text{HAS2}$ expression by keratinocytes.

Significance: Stimulation of hyaluronan synthesis is an inherent part of epidermal keratinocyte activation and injury response.

ABSTRACT

Hyaluronan, a major matrix molecule in epidermis, is often increased by stimuli that enhance keratinocyte proliferation and migration. We found that small amounts of UDP-sugars were released from keratinocytes and that UDP-glucose (UDP-Glc) added into keratinocyte cultures induced a specific, rapid induction of hyaluronan synthase 2 ($\text{HAS2}$), and an increase of hyaluronan synthesis. The upregulation of $\text{HAS2}$ was associated with JAK2 and ERK1/2 activation, and specific tyr705-phosphorylation of the transcription factor STAT3. Inhibition of JAK2, STAT3 or G$_i$-coupled receptors blocked the induction of $\text{HAS2}$ expression by UDP-Glc, the latter inhibitor suggesting that the signaling was triggered by the UDP-sugar receptor P2Y$_{14}$. Chromatin immunoprecipitations demonstrated increased promoter binding of p-tyr705-STAT3 at the time of $\text{HAS2}$ induction. Interestingly, at the same time p-ser727-STAT3 binding to its response element regions in $\text{HAS2}$ promoter was unchanged or decreased. UDP-Glc also stimulated keratinocyte migration, proliferation, and IL-8 expression, supporting a notion that UDP-Glc signals for epidermal inflammation, enhanced hyaluronan synthesis as an integral part of it.

INTRODUCTION

Hyaluronan is a large, ubiquitous glycosaminoglycan, consisting of alternating N-acetyl glucosamine (GlcNAc) and glucuronic acid (GlcUA) repeating units. It occupies the pericellular and extracellular space of many cell types, including basal and spinous cell layers of skin epidermis (1). It acts as a highly hydrated space filler, but also stimulates proliferation and migration through binding to its receptors, like CD44 and RHAMM (2-4). In epidermis, hyaluronan synthesis has been shown to increase rapidly in tissue activation, for example due to injury like epidermal wounding (5-8), presumably to help cell growth and movement to cover the wound. Hyaluronan disappears in stratum...
granulosum before terminal differentiation of the keratinocytes, and reduction of epidermal hyaluronan promotes differentiation (9, 10). Accordingly, stimulation of hyaluronan synthesis and increase of its content associates with compromised epidermal water barrier and morphologically incomplete differentiation (11). Moreover, hyaluronan increases in epidermal hyperproliferation and squamous cell cancer induced by UV-irradiation (12).

The three mammalian HAS isoforms are multispan transmembrane proteins. They are active when inserted in plasma membrane, transferring GlcNAc and GlcUA from the corresponding cytosolic UDP-sugars to the reducing end of the growing hyaluronan chain (13) that is extruded into extracellular space through a pore formed by the enzyme itself (14, 15). Among the three HAS genes HAS2 shows the highest expression in keratinocytes, and is upregulated by epidermal growth factor (EGF) (11), keratinocyte growth factor (KGF) (16), TNFa (17), interferon-γ (18) and all-trans-retinoid acid (19), whereas transforming growth factor β (TGFβ) downregulates its expression in keratinocytes (11). The regulation of HAS2 expression involves several transcription factors with functional response elements in its promoter. These include retinoic acid receptor (RAR), nuclear factor κB (NF-κB), cAMP response element binding protein 1 (CREB1), specificity protein 1 (SP1), yin-yang 1 (YY1), and STAT (20, 21). For example, EGF receptor activation enhances tyrosine 705-phosphorylated STAT3 binding to the promoter, inducing HAS2 gene expression (21).

The expression of HAS2 is also influenced by cellular supply of its own substrate UDP-GlcNAc, the abundance of which triggers a suppressive feedback loop mediated by the transcription factors SP1 and YY1. Their binding to HAS2 promoter is subject to regulation by their O-GlcNAc modification that is dependent on the cellular concentration of UDP-GlcNAc (22). Cytosolic UDP-GlcNAc has thus a double function; it stimulates hyaluronan synthesis as a crucial substrate of the HAS enzyme, and as a stabilizer of the HAS2 enzyme (23), but inhibits it through transcriptional suppression of the synthesis of HAS2 protein.

It has been recently confirmed that UDP-sugars exist also in the extracellular fluids (24), released by cellular injury or, as suggested recently, in a regulated fashion (24, 25). The idea of regulated secretion is in line with the finding that increasing UDP-sugar transport into Golgi apparatus stimulates UDP-sugar release through vesicular transport (24, 25). Interestingly, there is a G-protein-coupled purinergic plasma membrane receptor (P2Y14) specific for UDP-sugars (26), suggesting a biological signaling function for extracellular UDP-sugars. Release of UDP-sugars might thus serve as an autocrine or paracrine signaling system. The system may serve as a warning signal after tissue injury, since thrombin has been shown to stimulate the release of UDP-Glc (24), and receptor binding of UDP-Glc induces the expression of IL-8, a mediator of inflammation (27). Most potent agonist of the P2Y14 is UDP-Glc (26).

P2Y14 has a relatively wide distribution in human tissues, with highest expression levels in placenta, adipose tissue, stomach, and intestine, and moderate levels in the brain, spleen, lung and liver (28). P2Y14 is an important regulator of mesenchymal differentiation, especially adipogenesis (29). Activation of P2Y14 receptor by UDP-Glc promotes MAP kinase signaling (30) and mobilizes intracellular Ca2+ stores (27). Extracellular UDP-Glc promotes IL-8 secretion (27) and stimulates mast cell degranulation (31). Keratinocytes express several subtypes of P2Y receptors (32), known to regulate their proliferation and differentiation (33). However, nothing is known about the function P2Y14 receptor and extracellular UDP-sugars in keratinocytes. In this work we show that extracellular UDP-Glc stimulates HAS2 expression, hyaluronan synthesis, proliferation and migration of cultured human keratinocytes. The upregulation of HAS2 is mediated through a Gi-linked P2Y receptor and extracellular UDP-sugars in keratinocytes. In this work we show that extracellular UDP-Glc stimulates HAS2 expression, hyaluronan synthesis, proliferation and migration of cultured human keratinocytes. The upregulation of HAS2 is mediated through a Gi-linked P2Y receptor, most likely P2Y14, and phosphorylation of JAK and STAT3, the latter specifically in tyrosine-705, which correlates with its binding to HAS2 promoter after UDP-Glc treatment.

**EXPERIMENTAL PROCEDURES**

Cell culture - The human immortalized epidermal keratinocyte cell line HaCaT (34) was cultured in DMEM (Sigma, St.Louis, MO) supplemented with 10% FBS (Hyclone, Logan, UT), 2 mM L-glutamine (Euroclone, Milan, Italy),
50 units/ml penicillin and 50 µg/ml streptomycin (Euroclone). UDP-Glc was purchased from Sigma.

RNA extraction and QPCR - QPCR analyses were done from total RNA extracted with Eurozol (Euroclone) after cDNA synthesis with Verso cDNA kit (Thermo Fischer, Waltham, MA), on an MX3000P thermal cycler (Stratagene, La Jolla; CA), using the Fast Start universal SYBR Green Master (ROX) (Roche, Basel, Switzerland). Fold inductions were calculated using the formula $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t(\text{sample}) - \Delta C_t(\text{non-treated replicate1})$, $\Delta C_t$ is $C_t(\text{gene of interest}) - C_t(\text{ARPO})$ and the $C_t$ is the cycle at which the threshold is crossed. The gene-specific primers for the genes analyzed are shown in Table 1. PCR product quality was monitored using post-PcR melt curve analysis.

Enzyme-linked sorbent assay (ELSA) for hyaluronan - Media from the cultures were assayed for the concentration of hyaluronan using a sandwich-type enzyme linked sorbent assay (ELSA) as described previously (35). The hyaluronan released into the media was normalized per 10,000 cells, counted by using a hemocytometer.

Anion-exchange HPLC of UDP-Glc - Cells were cultured until 80% confluence. After treatments, cells were counted from one plate for normalization of the results while cells and media from parallel plates were used to measure UDP-Glc.

Cells were washed with cold PBS on ice. Cold acetonitrile was added to precipitate proteins and extract UDP-sugars. Cells were scraped off and the acetonitrile with cell debris was transferred into centrifuge tubes, the plates washed with 1 ml of deionized water and combined with the first extract, centrifuged at 6000xg for 20 min, the supernatants transferred to clean tubes, evaporated in a vacuum centrifuge, and dissolved in PBS for further purification by solid phase extraction on Superclean Envi-Carb SPE cartridges (Sigma) as described previously (36).

Culture media were rapidly frozen, lyophilized and redissolved in 250 µl of water. Salts and glucose were separated from UDP-sugars by Superdex Peptide® column eluted at 1 ml/min with 12 mM NH₄HCO₃. The fractions containing UDP-sugars (prechecked using UDP-Glc standard) were combined and purified further with Superclean Envi-Carb SPE, as above.

Purified samples were evaporated by vacuum centrifugation and dissolved in 300 µl of water for anion-exchange HPLC on a CarpoPac™ PA1 column (4 x 250 mm, Dionex, Sunnyvale, CA). The column was eluted at 1 ml/min with a gradient made of ultrapure H₂O (A), 1.3 M sodium-borate pH 7.0 (B), 1M sodium acetate, pH 7.0 (C), and 1.5 M sodium-borate pH 7.5 (D). The column was equilibrated with 45%/55%/0%/0% (v/v/v/v) of buffers A, B, C and D. Elution was performed with the following program: T0=45%/55%/0%/0%, T23=40%/55%/0%/5%, T48=36%/55%/0%/9%, T65=20.6%/12.3%/61.8%/5.3%, T69=20.6%/12.3%/61.8%/5.3%, T70=10.4%/12.3%/72%/5.3%, T82=45%/55%/0%/0%, T88=45%/55%/0%/0%. Integrated peak areas were calculated and compared to those of standard nucleotide sugars.

Proteome Profiler™ Array – HaCaT cells were incubated for 30 min in medium supplemented with 100 µM UDP-Glc. Cells were washed with PBS and cell lysis and protein extraction performed as described in the protocol of Proteome profiler™ Array, Human phospho-kinase array kit (R&D systems, Minneapolis, MN).

Western blotting - Proteins were extracted on ice with RIPA lysis buffer (PBS, pH 7.4 with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml sodium orthovanadate, and 1% phosphatase inhibitor cocktail 2, and 0.5% protease inhibitor cocktail (Sigma). The samples (20 µg protein) were resolved by 10% SDS-PAGE, followed by transfer onto nitrocellulose membrane (Whatman®, Dassel, Germany) by 35 mA/cm² constant current with a semidy blotter (Biometra, Göttingen, Germany). The membrane was placed in a SNAP blotting system (Millipore, Bedford, MA), blocked with 1% BSA, washed with Tris-buffered saline (TBS), 0.1% Tween, and incubated with the primary antibodies: phospho-JAK2 (Tyr1007/1008), phospho-STAT3 (tyr705) 1:1000, phospho-STAT3 (ser727) 1:1000 (all from Cell Signaling, Danvers, MA), phospho-ERK 1:500 (Santa Cruz) and β-actin 1:2500 (Sigma). After washes, the membrane was incubated with the fluorescent secondary antibodies anti-rabbit 680 and anti-mouse 800, 1:5000 (Pierce, Rockford, IL). Protein bands were visualized and quantified with Odyssey® infrared imaging system (Li-Cor
Bioscience, Lincoln, NE). The results represent the ratio of band intensities between the protein of interest and β-actin.

Signaling inhibitors - Cells were treated with pertussis toxin (PTX, 100 ng/ml, Tocris Bioscience, Southampton, UK) an inhibitor of G_i-coupled receptors, for 17 h prior to UDP-Glc addition. Cells were treated for 2h with 30 μM AG490 (Sigma) an inhibitor of JAK2, 50 μM STAT3 inhibitor IX (Calbiochem, Darmstadt, Germany), 0.5-2 μM PD98059 (Calbiochem) and 0.5-2 μM UO126 (Calbiochem), inhibitors of MEK1/2, before adding UDP-Glc. PTX was dissolved in water, AG490 in ethanol and STAT3 inhibitor IX, PD98059, and UO126 in DMSO. Equal amounts of those solvents were used as controls.

Chromatin immunoprecipitation - After 2 h incubations with or without 100 μM UDP-Glc, nuclear proteins were cross-linked to DNA by adding formaldehyde directly to the medium to a final concentration of 1% for 10 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 0.15 M and incubating for 10 min at room temperature.

The medium was removed and the cells were washed twice with ice-cold PBS. The cells were then collected in ice-cold PBS supplemented with a protease inhibitor cocktail (Sigma). After centrifugation, the cell pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors, 50 mM Tris-HCl, pH 8.1) and incubated for 10 min at room temperature. The lysates were sonicated, until DNA fragments of 300 to 1000 bp in length were obtained (in preliminary tests).

Cellular debris was removed by centrifugation. At this step 20 μl of the supernatant was taken as the input sample and diluted 1:5 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, protease inhibitors, 16.7 mM Tris-HCl, pH 8.1). The rest of the supernatant was divided into the aliquots which were diluted 1:10 in ChIP dilution buffer and incubated with the indicated antibodies (1:100-1:400) overnight at 4 °C with rotation. The antibodies against rabbit IgG (sc-2027), pSTAT3 (Tyr705) (sc-7993) were obtained from Santa Cruz Biotechnologies and pSTAT (Ser727) (#9134) and total STAT3 (#9132) were obtained from Cell Signaling.

The immunocomplexes were collected with 20 μl of protein G-magnetic beads (Millipore) for 1 h at 4 °C with rotation. The beads were separated from the supernatant using a magnetic rack (Qiagen, Valencia, USA). The pellets were washed sequentially for 3 min by rotation with 1 ml of each of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1) and LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Finally, the beads were washed twice with 1 ml of TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.1).

The immunocomplexes were eluted by adding 350 μl of elution buffer (1% SDS, 100 mM NaHCO3), incubating at 65°C for 30 min and separating the beads from the supernatant on magnetic rack. This 350 μl sample was the enriched output sample. The crosslinking was reversed and the remaining proteins were digested by adding 1.8 μl of proteinase K (final concentration 10 μg/μl, Fermentas) to both the input and output samples, and incubating overnight at 65°C. The DNA was recovered by using QIAquick® PCR purification kit (Qiagen).

The DNA was used as a template for PCR with the following profile: 10 min preincubation at 95 °C, and 45 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 30 s elongation at 72 °C, with one final incubation for 10 min at 72 °C. The primers for the amplification are presented in Table 2. The resulting PCR products were quantified and expressed as percent of the input control. Fold changes were calculated using the formula $2^{(ΔCt)}$, where $ΔCt$ is Ct output - Ct input and Ct is the cycle at which the threshold is crossed. The results are presented as fold changes over the value of samples precipitated with normal rabbit IgG. Input samples were diluted 13.3 times compared with outputs.

Proliferation and migration - For the proliferation assay 20,000 HaCaT cells were seeded on a 24-well plate and after 4 h UDP-Glc was added to a final concentration of 100 μM. The media containing UDP-Glc was replaced each day. Cells were detached with trypsin-EDTA at 1-5 days following plating and counted with hemocytometer.

Cell proliferation was also studied by BrdU-staining. HaCaT cells were seeded on 8-well
chamber slides (Nalge Nunc, Naperville, IL), cultured to 80% confluence, and treated with 100µM UDP-Glc overnight. Next day the cultures were incubated with 5-bromo-2′deoxyuridine (1:1000, BrdU, Roche, Penzberg, Germany) in DMEM for 1 h, washed with PBS, and fixed with 4% paraformaldehyde on ice. The slides were washed with 100 mM Na-phosphate, pH 7.4 (PB), incubated with 70% EtOH for 10 min, and washed with PB again. The slides were treated with 2 M HCl for 30 min at 37°C, washed with PB, and blocked with 1% BSA-PB for 10 min at room temperature, then immunostained with the anti-BrdU antibody (1:500, Sigma-Aldrich) in 1% BSA-PB for 2 h at 37°C, washed with PB, and incubated with biotinylated anti-mouse antibody (1:200, Vector Laboratories, Burlingame, CA) in 1% BSA-PB for 30 min at 37°C, and washed with PB. The bound antibodies were visualized with the avidin-biotin peroxidase (1:200, ABC-standard kit, Vector) in PB for 1 h at room temperature, washed with PB and incubated with 0.05% 3,3′-diaminobenzidine (DAB) and 0.03% hydrogen peroxide for 5 min. The nuclei were stained with hematoxylin. The cells were mounted using Supermount (BioGenex, San Ramon, CA) The number of nuclei showing positive staining and the number of all nuclei were counted from 10 fields, randomly photographed using a 10x objective.

For studies on apoptosis HaCaT cells were seeded on 96-well plates (Cell Star®, Greiner Bio-One, Kremsmunster, Austria). Next day, fresh medium containing 100 µM UDP-Glc was changed. After a 24 h incubation, living and dead cells were determined with a kit according to the instructions of the manufacturer (CytoTox-Glo™ Cytotoxicity Assay, Promega, Fitchburg, WI).

Extracellular UDP-Glc also slightly increased the expression of HAS3, which was not further analyzed, while HAS1 expression was too low for reliable analysis. To see if the rise in HAS2 mRNA expression was reflected in hyaluronan secretion, its content in the culture medium was assayed. A significant, 175% rise in the amount of hyaluronan was found in samples collected 6 h after the addition UDP-Glc (Fig. 1B). This change was not observed in samples collected at the 4 h time point. The delay in the increased hyaluronan production was in line with an idea that transcriptional activation preceded the increased hyaluronan production.

**RESULTS**

Extracellular UDP-Glc stimulates HAS2 expression and induces hyaluronan synthesis - A signaling function has been established for extracellular UDP-sugars, activating several intracellular pathways and modulating cell functions. Given the importance of the intracellular UDP-sugars for hyaluronan synthesis (35, 37, 23) and HAS expression (22, 38) we examined whether UDP-sugars are also active when added in the extracellular compartment. Introduction of UDP-Glc induced a clear increase of HAS2 mRNA while UDP-GlcUA caused a modest increase, and UDP-Gal and UDP-GlcNAc did not present significant effects (Fig. 1A). The activation of HAS2 expression was rapid, with a significant increase already 90 min after the addition of UDP-Glc, while the expression level had returned back to control level after 6 h (Fig. 1C). The expression of HAS2 showed a steep increase between 10 and 50 µM, but without significant further increase above 100 µM, as if crossing a threshold concentration would trigger a rapidly saturable response (Fig. 1D). UDP-Glc also slightly increased the expression of HAS3, which was not further analyzed, while HAS1 expression was too low for reliable analysis. To see if the rise in HAS2 mRNA expression was reflected in hyaluronan secretion, its content in the culture medium was assayed. A significant, 175% rise in the amount of hyaluronan was found in samples collected 6 h after the addition UDP-Glc (Fig. 1B). This change was not observed in samples collected at the 4 h time point. The delay in the increased hyaluronan production was in line with an idea that transcriptional activation preceded the increased hyaluronan production.

Statistical analysis - Normally distributed data were analyzed by paired samples t-tests when comparing control and UDP-Glc treated cultures, and by one-way analysis of variance with Dunnet’s post hoc test, when many treatments were tested.
Glc in the medium varied between 1 and 2 pmol/10,000 cells.

We also studied how the addition of UDP-Glc influenced its intracellular content. 30 min after addition of 100 µM of UDP-Glc to the culture medium there was already a 1.8-fold increase, and at 2 h time point the intracellular level of UDP-Glc was increased by 2.7-fold (Fig. 2B), suggesting that a part of the medium UDP-Glc was endocytosed.

The effect of UDP-Glc on HAS2 transcription is mediated by G_i-coupled P2Y receptors - UDP-Glc has been shown to act as an agonist of the G_i-coupled receptor P2Y_{14} (25). Three G_i-coupled P2Y receptor subtypes have been identified, namely P2Y_{12}, P2Y_{13} and P2Y_{14}. ADP activates the former two, and P2Y_{14} is the only subtype known to be activated by UDP-Glc (39). P2Y_{14} is expressed in HaCaT cells (32, 40). To study the involvement of P2Y_{14}, experiments were done in cells pretreated with pertussis toxin (PTX), which specifically inhibits signal transduction through G_i-coupled receptors by catalyzing the ADP-ribosylation of the a subunit of G_i protein, thereby uncoupling the G protein from receptor (41). We used 100 ng/ml PTX, previously reported to effectively block G_i-coupled receptors (31). PTX did not show toxic effects on HaCat culture, as indicated by cell morphology and the absence of increased apoptosis in the cytotoxicity assay, but it significantly inhibited the stimulation of the HAS2 expression evoked by UDP-Glc (Fig. 3A), indicating that UDP-Glc signaling involves G_i-coupled receptors, very likely P2Y_{14}.

For background, we checked whether UDP-Glc also induces signals other than those leading to HAS2 expression. As enhanced secretion of IL-8 was previously reported in airway epithelial cells treated with UDP-Glc (27), we measured its expression in HaCaT cells. Indeed, IL-8 mRNA was significantly upregulated by UDP-Glc simultaneously with HAS2 (Fig. 3B), indicating a wider range of signals activated by UDP-Glc in HaCaT cells. The induction of IL-8 expression was also blocked by PTX (Fig. 3C). The findings support the idea that the HAS2 response is a part of an inflammatory reaction.

**UDP-Glc stimulates ERK and STAT3 phosphorylation** - To study which signaling pathways are involved in the upregulation of HAS2 expression, we used a phosphokinase array (Fig. 4A). After 30 min incubation of the HaCaT cells with UDP-Glc, the most interesting findings were tyr705 phosphorylation of STAT3, an important transcriptional regulator of HAS2 (11, 21) and its upstream activator ERK (42, 43). The increase of ERK and STAT3 tyr705 phosphorylations by UDP-Glc was confirmed by western blotting (Fig. 4B, C).

Since p-ERK can activate STAT3, we checked whether inhibition of p-ERK blocks the upregulation of HAS2 by UDP-Glc. It turned out that UO126 which totally abolished ERK phosphorylation (Fig. 5A), efficiently cut the basal expression of HAS2 down to ~30% of control, while the relative stimulation by UDP-Glc was minimally affected (Fig. 5A,B). This finding was reproduced by PD98059, another inhibitor of ERK phosphorylation (Fig. 5A,C). The higher efficiency of UO126 over PD98059 has been noted before (44) and may be due to the fact that UO126 targets both MEK1 and MEK2, while the main target of PD98059 is MEK1. Data on both inhibitors and their both concentrations were subjected to statistical analysis which indicated that UDP-Glc activation of HAS2 remained significant in the presence of ERK inhibition. This suggests that STAT3 was mainly activated by another signaling pathway.

**JAK2 phosphorylation associates to UDP-Glc-stimulated HAS2 expression** – Besides ERK, Janus kinase 2 (JAK2) has been found to activate STAT3 (45). We therefore examined the level of p-1007/1008JAK2 following introduction of UDP-Glc. There was a transient increase of JAK2 at 10-20 min, followed by return to control level at 30-60 min (Fig. 6A,B). The activation suggested that JAK2 could also be involved in the upregulation of HAS2. The contribution of JAK2 was supported by the decrease of HAS2 mRNA with AG490, an inhibitor of JAK2 (Fig. 6C).

**UDP-Glc causes sequencial activation of STAT3 at tyr705 and ser727** – STAT3 has two phosphorylation sites (tyr705 and ser727) commonly associated with its activation (45). Time-dependent changes of these two phosphorylations were analyzed with western blotting to correlate their levels with the transient
increase in HAS2 mRNA. p-tyr705STAT3 increased at 30 min, reached its maximum at 60 min and declined close to control level at 120 min (Fig. 7A,B), thus preceding the peak of HAS2 at 120 min (Fig. 1C). In contrast, there was no significant change in the level of p-ser727 at 30 and 60 min, while a significant increase was observed at 120 min (Fig. 7A, C). This suggests that the increase of HAS2 mRNA fits better with STAT3 phosphorylation of p-705 than p-727.

The tyr705 phosphorylation of STAT3 was very efficiently blocked by STAT3 inhibitor IX (Fig. 7D). The JAK2 inhibitor AG490 also blocked the UDP-Glc-induced increase of p-tyr705STAT3 (Fig. 7D), suggesting that JAK2 was involved in the p-tyr705STAT3 activation. The increase of UDP-Glc-induced HAS2 expression was completely neutralized with STAT3 inhibitor IX, although the inhibitor itself increased the basal level of HAS mRNA (Fig. 7E).

Together, these results suggest that the early JAK2 activation triggers tyr705 phosphorylation of STAT3, which is followed by an increase of HAS2 mRNA.

Tyr705 phosphorylated STAT3 binds to HAS2 promoter after UDP-Glc treatment - Since the data suggested involvement of STAT3 in the UDP-Glc-induced HAS2 response, and this transcription factor is known to have functional binding sites on HAS2 promoter, we studied if UDP-Glc-stimulated signaling increases STAT3 binding to the promoter regions with reported STAT response elements (21) and/or to the transcription start site (TSS). After 2 h incubation with UDP-Glc, STAT3 binding to the TSS containing region was significantly increased (Fig. 8A). Separate ChIP analyses with p-tyr705 and p-ser727STAT3 antibodies revealed that the increase of STAT3 binding to TSS containing region was solely due to the p-tyr705 STAT3 while there was no change in the binding of p-ser727STAT3 (Fig. 8A). Similar findings were made in the three other regions of the HAS2 promoter with STAT response elements, showing increased p-tyr705 STAT3 binding and no change or reduced binding of p-ser727 STAT (Fig. 8B-D).

Taken together, the data indicate that UDP-Glc, through a G_i-coupled P2Y_14 receptor and JAK2 activation stimulates tyr705-phosphorylation of STAT3, leading to its binding to HAS2 promoter and stimulation of its transcription, resulting in enhanced hyaluronan synthesis.

UDP-Glc stimulates cell proliferation and migration – Since enhanced migratory and proliferative activity of epidermal keratinocytes is a common feature in inflammation (46) and often associated with elevated hyaluronan synthesis (2, 47), we studied whether UDP-Glc stimulates these cellular functions. In time series experiments daily UDP-Glc treatment did not influence cell numbers until day 5, when the cells approached confluence (Fig. 9C). At that time a small (15%), but statistically significant increase in cell counts was observed. To check if the late UDP-Glc response was due to slow or indirect mechanisms, or was related to confluence, we treated near confluent cultures with UDP-Glc for 24 h and analyzed the number of proliferating cells with BrDU-labeling. A small (12%) but consistent increase in the number BrDU-positive cells was observed in UDP-Glc treated cultures (Fig. 9A), suggesting that the proliferative response may relate to the confluency or differentiation status of the cells. UDP-Glc treatment did not influence the number of dead cells (Fig. 9B).

In a scratch wound assay, HaCaT cells treated with UDP-Glc for 24 h migrated significantly more than the untreated cells (Fig. 9D). Although the difference between the means was rather modest (~13 %), the result was reproduced in all experiments performed.

To summarize these results, UDP-Glc does not induce apoptosis, and it modestly increases cell proliferation and migration.

DISCUSSION

The present study established that HAS2 gene expression in keratinocytes is subject to regulation by extracellular UDP-sugars, complementing the recently described control by intracellular UDP-sugars (22). However, the extracellular and intracellular signaling mechanisms appear to be completely independent. The intracellular regulation comes through the content of UDP-GlcNAc, controlling O-GlcNAc modifications in the transcription factors SP1 and YY1, and the HAS2 enzyme itself (23). In contrast, among the different UDP-sugars UDP-Glc is the dominant
extracellular effector on HAS2 expression, mediated by G\textsubscript{i}-coupled P2Y receptors, most likely P2Y\textsubscript{14}, leading to JAK2 activation and tyr705 phosphorylation of the transcription factor STAT3, and its binding to HAS2 promoter. The resulting increase of hyaluronan synthesis occurred together with enhanced migration and proliferation, phenotypic changes previously found to accompany the accumulation of hyaluronan by other keratinocyte activators like EGF (2), KGF (16) and retinoic acid (19).

The UDP-Glc stimulation on HAS2 expression comes up very rapidly. Because the HA-response requires more time, it is not likely to result from direct regulation of HAS activity, but rather be a result of increased mRNA level. JAK2-STAT3 signaling appeared to be involved in HAS2 response suggesting transcriptional regulation, although changes in mRNA stability are also possible. HAS2 response also and also recedes fast, probably due to the fast decay of UDP-Glc in the growth medium. Ectonucleotidases are known to catalyze hydrolysis of nucleotides on the outer surface of plasma membrane, and in the extracellular milieu (48), which can explain the observed decay of the UDP-Glc.

The increase of intracellular UDP-Glc during the incubation suggests that a part of UDP-Glc is taken up into the cells, where it remained at an elevated level for at least 6 h. This finding is in line with the reported recycling of nucleotides between extracellular and intracellular compartments (48) but no data exist on the mechanisms of UDP-Glc uptake. Being membrane impermeable, and lacking known plasma membrane transport channels, UDP-Glc can be taken up by fluid phase endocytosis (49), with or without help by receptors. The increased cellular UDP-Glc is perhaps located in endosomes, ER or Golgi apparatus. However, we believe that the cellular uptake is not a major mediator of signals induced by extracellular UDP-Glc, considering the fact that inhibition of the UDP-Glc receptor with Pertussis toxin abolished its stimulatory effect on HAS2 expression.

UDP-Glc is an activating ligand of the G\textsubscript{i}-coupled receptor P2Y\textsubscript{14}. The other G\textsubscript{i}-coupled P2Y receptor subtypes are activated by ADP (39). P2Y\textsubscript{14} involvement was strongly supported by the notion that PTX, a potent inhibitor of G\textsubscript{i}-coupled receptors, prevented the UDP-Glc-induced upregulation of HAS2 expression. RT-PCR also confirmed that P2Y\textsubscript{14} was expressed in the HaCaT cells.

It has been reported that activation of G\textsubscript{i}-coupled receptors by extracellular UDP-Glc leads to activation of ERK (30), JNK and p38 MAP kinases (31), and mobilization of intracellular Ca\textsuperscript{2+} stores (27). In agreement with the reports (30), HaCaT cultures showed elevated ERK phosphorylation after UDP-Glc treatment. However, inhibition of the ERK signaling pathway (by U0126 and PD98059) did not block the UDP-Glc-induced stimulation of HAS2 expression (Fig. 4C, D), indicating that other pathways were involved in the HAS2 response. Indeed, phosphorylation of JAK2, often activated by inflammatory cytokines, was increased by UDP-Glc, and an inhibitor of JAK2 reduced HAS2 upregulation, suggesting that JAK2 was a more important contributor to the response than ERK1/2.

The STAT transcription factors are the established downstream mediators of JAK2 signaling, governing the expression of a number of genes, especially those involved in cell proliferation (50). Given the fact that the P2Y-group receptors have been shown to activate STAT3 (51), and keratinocytes present functional STAT3 response elements on their HAS2 promoter (21), it was not unexpected that UDP-Glc activated STAT3, and a STAT3 inhibitor blocked the UDP-Glc-induced HAS2 expression. It is thus very likely that the G\textsubscript{i}-coupled receptor activation signaled the upregulation of HAS2 expression through the JAK2-STAT3 pathway.

On the other hand, the strong downregulation of the basal HAS2 expression by the inhibitors of the MAPK-pathway is in line with previous findings that growth factors and cytokines in the serum activate its transcription (38) and the inhibitors block this part of the HAS2 expression. Although we cannot completely exclude unspecific effects of the inhibitors, it was clear that during the 4 h incubation no obvious signs of toxicity or increased apoptosis was detected with any of them. The STAT response elements on HAS2 promoter bind p-tyr705 STAT3 and activate the gene (21). Our data show that 2 h after introduction of UDP-Glc, when HAS2 mRNA reached its peak level, HAS2 promoter binding of both total STAT3 and p-tyr705STAT3 are increased, whereas at the same time p-ser727
STAT3 tended to vanish from the chromatin immunoprecipitates. It has been reported that tyr705 phosphorylation of STAT3 is dependent on JAK activity, whereas ERK and other MAP-kinases have been suggested to promote phosphorylation of ser727 (42, 43). Phosphorylation of tyr705 causes dimerization of STAT3, which leads to its nuclear localization (52).

The role of ser727 phosphorylation of STAT3 is controversial at the moment. It has been reported to promote the transcriptional activity of STAT3 by recruiting coactivators (53-55). On the other hand, it may enhance tyr705 dephosphorylation, and thereby inhibit transcription (56). The reciprocal changes we found in tyr705 and ser727 phosphorylations of STAT3 are in line with the latter report, supporting the role of tyr705 as an activating modification (56). Of course, the function of ser727 phosphorylation may depend on the specific gene and cell type.

How UDP-sugars get into the extracellular space has remained obscure. Plasma membrane defect following cellular injury is one potential source. On the other hand, the positive correlation between UDP-sugar transporter activity in Golgi membrane, and the UDP-Glc appearance in culture medium has been suggested to indicate its controlled secretion, likely by vesicular transport to plasma membrane (25). The concentration of UDP-Glc in astrocytoma culture medium is quite small but is increased by thrombin treatment (24), also suggesting regulated secretion. The concentration of UDP-Glc was quite small in the growth medium of keratinocytes. However, it is very difficult to estimate the effective concentration in vivo, considering the minor intercellular space between epidermal keratinocytes (57), the labile nature of the substance, and the unknown mechanism of its export.

Extracellular UDP-Glc has been related to many cellular functions. Involvement in inflammation is suggested by its ability to induce the secretion of IL-8 in airway epithelial cells (27) and degranulation of mast cells via activation of P2Y14-receptor (31). In rat brain the expression of P2Y14 receptor level is upregulated by a challenge with LPS (58, 59), suggesting a role in reactive astrogliosis. In addition to inflammation, extracellular UDP-Glc and P2Y14 receptor have been associated to the commitment of mesenchymal stem cells to adipogenic and osteogenic differentiation (29), and to the chemotaxis of hematopoietic stem cells (60). Our results suggest that extracellular UDP-Glc has a functional role also in skin epidermis. The induction of HAS2 and IL-8 gene expressions, and the stimulation of migration and proliferation, suggest that its biological function may be to contribute to epidermal activation as a response to trauma or inflammation.

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**FOOTNOTES**

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The abbreviations used are: HABC, hyaluronan binding complex of the cartilage aggrecan G1 domain and link protein; HAS, hyaluronan synthase; UDP-GlcNAc, UDP-N-acetyl glucosamine; UDP-GlcUA, UDP-Glucuronic acid; UDP-Glc, UDP-Glucose; P2Y<sub>14</sub>, G<sub>i</sub>-protein-coupled purinergic receptor specific for UDP-sugars; PTX, Pertussis toxin.

**FIGURE LEGENDS**

**FIGURE 1.** Extracellular UDP-sugars regulate *HAS2* expression and hyaluronan synthesis. (A) HaCaT cultures were treated for 2h with 100 µM of the indicated UDP-sugars and analyzed for *HAS2* expression. (B) UDP-Glc as the most powerful stimulator was used to study hyaluronan release into growth medium, analyzed 4 and 6 h after adding UDP-Glc. (C) The time course of *HAS2* induction using 100 µM concentration of UDP-Glc. (D) Effect of UDP-Glc concentration on *HAS2* expression during a 2 h incubation. In panels A and C the data represent means±SE of three independent experiments, each done as duplicates. The statistical significances of the differences between control and UDP-sugars were tested using Dunnet’s test. In panels B and D the data represent means±SE of 5 and 4 independent experiments, respectively. Statistical significance was estimated using paired sample t-test. *p<0.05; **p<0.01 and ***p<0.001.

**FIGURE 2.** Added UDP-Glc decreases in the HaCaT culture medium and increases in the cells. HaCaT cells were incubated for 1, 30, 120 and 360 min with 100 µM UDP-Glc. (A) extracellular and (B) intracellular UDP-Glc was analyzed with anion exchange HPLC, with means±SE of three and four independent experiments, respectively. In (A) the time point 1 min was taken as the control (100%), indicating the starting level of the UDP-Glc in the medium. In (B) the time point 0 (100%) indicates the average intracellular UDP-Glc level in non-treated cultures. The statistical significance between non-treated and UDP-Glc-treated cultures at each time point indicate the results of Dunnet’s test: *p<0.05; **p<0.01 and ***p<0.001.

**FIGURE 3.** Pertussis toxin inhibits UDP-Glc-induced expression of *HAS2* and *IL-8*. HaCaT cells were preincubated overnight with or without 100 ng/ml pertussis toxin (PTX) before UDP-Glc was added to 100 µM final concentration and incubation continued for 2 h. (A) *HAS2* and (B, C) *IL-8* mRNA were measured by QRT-PCR. Data represent means±SE of four experiments, *** p<0.001, Dunnet’s test (A), of five experiments, *p=0.017, paired sample t-test (B) and in chart (C) means±range of one experiment with replicate samples.
FIGURE 4. UDP-Glc activates ERK and STAT3. (A) Dot blot images from a phosphokinase array on protein samples extracted from cells incubated for 30 min with 100 μM UDP-Glc. The phosphorylated proteins STATtyr705 and ERK are encircled. Other spots in array: A3A4: p38a, A7A8: JNK1/2/3, A9A10: GSK-3a/b, B5B6: MSK1/2, B9B10: Akt1/2/3, C1C2: TOR, C3C4: CREB, C7C8: AMPKa2, C9C10: b-catenin, D1D2: Src, D7D8: STAT2, D9D10: STAT5a, E1E2: Fyn, E3E4: Yes, E9E10: STAT5b, F1F2: Hck, F3F4: Chk-2, F5F6: PDGF Rb, F9F10: STAT5a/b. (B, C) Western blots of cells incubated for 30-60 min with 100 μM UDP-Glc with antibodies against p-ERK and p-tyr705STAT3. Means±SD of four independent experiments are shown. Statistical significance of the difference vs. control, ** p=0.002 (Dunnet’s test).

FIGURE 5. ERK contributes to basal HAS2 mRNA level but not UDP-Glc-mediated upregulation. (A) Phosphorylated ERK (pERK) levels after 120 min preincubation with MEK inhibitors: UO126 and PD98059 followed 30 min incubation with or without 100 μM UDP-Glc. (B, C) Cells preincubated for 120 min in the presence of the UO126 and the PD98059, were incubated for 60 min with each inhibitor with or without 100 μM UDP-Glc. HAS2 expression was assayed and compared between UDP-Glc-treated and untreated cultures, the relative increase indicated by the percentages above the columns. Means±SD of 2-3 independent experiments are shown. Analysis of variance indicated that the inhibitors did not influence the increase caused by UDP-Glc. In addition, UDP-Glc vs. control in the presence of the inhibitors, p<0.001 (t-test).

FIGURE 6. JAK2 phosphorylation associates with UDP-Glc-stimulated HAS2 expression. (A) Western blots of pJAK2 at the indicated times following introduction of 100 μM of UDP-Glc. (B) Quantitation of the western blots from 4-6 separate experiments at each time point, normalized to β-actin. *p<0.05, UDP-Glc vs. control, by paired sample T-test. (C) Effect of the JAK2 inhibitor AG490 (30 μM) on HAS2 expression in cultures treated with or without 100 μM UDP-Glc. The cultures were preincubated for 120 min with the inhibitor, followed by 120 min with or without 100 μM UDP-Glc. The data represent means±SE of 5 independent experiments. Statistical significance, control vs. UDP-Glc: ***p<0.001 (Dunnet’s test). (D) Verification of AG490 effect on JAK2 phosphorylation. The cultures were preincubated for 120 min with the inhibitor, followed by 120 min (pJAK) with or without 100 μM UDP-Glc.

FIGURE 7. UDP-Glc induces both p-tyr705 and p-ser727 phosphorylation of STAT3. (A) Western blots of p-tyr705STAT3 and p-ser727STAT3 30-120 min after introduction of 100 μM UDP-Glc. (B) Quantitation of the changes in phosphorylation of p-tyr705 and (C) p-ser727 of STAT3. Means±SE from 3-6 separate experiments are shown. **p<0.01, UDP-Glc vs. control, by paired sample T-test. (D) Verification of the effect of STAT3 inhibitor IX and the JAK2 inhibitor AG490 on STAT3 tyr705 phosphorylation after 120 min preincubation with the inhibitors followed by 60 min incubation with UDP-Glc. (E) Inhibition of the UDP-Glc-induced HAS2 upregulation by STAT3 inhibitor IX. The cultures were preincubated for 120 min with 50 μM STAT3 inhibitor IX, followed by 120 min in the presence and absence of 100 μM UDP-Glc. Means±SE of five separate experiments, *p<0.05, Dunnet’s test.

FIGURE 8. UDP-Glc treatment enhances p-tyr705-STAT3 binding to the HAS2 gene promoter. HaCaT cells were incubated for 2 h with 100 μM UDP-Glc and STAT3 binding into HAS2 promoter was studied by ChIP. Total STAT3, p-tyr705-STAT3 and p-ser727-STAT3 binding to the TSS region is shown in (A), to region -481 to -244 in (B), to region -1048 to -655 in (C) and to region -1896 to -1554 in (D). The data represent means±SE of five independent experiments. Statistical significance control vs. UDP-Glc: *, p<0.05 (paired sample t-test).
FIGURE 9. UDP-Glc stimulates HaCaT cell proliferation and migration. (A) Cell proliferation was studied by incubating HaCaT cells for 24 h with 100 µM UDP-Glc and then 1 h with BrdU. The specimens were processed for histology, immunostained for BrdU, and the percentage of BrdU-positive cells per all cells was calculated. (B) Apoptosis after 24h incubation with 100 µM UDP-Glc was studied by a cytotoxicity assay –kit. (C) Cell numbers were counted in a hemocytometer following incubations in 100 µM UDP-Glc for 1-5 days. A new culture medium, including UDP-Glc, was changed every day. (D) Cell migration was studied in a scratch wound model for 24 h in presence of 100 µM UDP-Glc. Data in (A) represent means±SE of four, (B) means±range of two and (C, D) means±SE of five independent experiments. Statistical significance, control vs. UDP-Glc *p<0.05; **p<0.01 (paired samples t-test).

Table 1. Primer sequences for QPCR of the reverse transcribed human genes.

| Gene name | Primer sequence (5´to 3´) |
|-----------|----------------------------|
| ARPO      | For- AGATGCAGCAGATCCGCAT   |
|           | Rev- GTGGTGTACCTAAAGCCTG   |
| HAS2      | For- CAGAATCCAAACAGACAGTTC |
|           | Rev- TAAGGTGTGTGTGTGACTGA  |
| IL-8      | For- GAGTGGACCACACTGCGCCA  |
|           | Rev-TCCACAAACCCTCTGCACCAGTT|
| P2Y14     | For- TCAGCAGATCATTCCTGTGC  |
|           | Rev-GGCTCATTCAAAAGTCAGCA   |

Table 2. Sequence and location of the PCR primers used in the ChIP assays of human HAS2 gene promoter.

| Region | Location | Primer sequence (5´to 3´) |
|--------|----------|---------------------------|
| 1 (TSS) | -32 to +57 | For- GGAGGCAGAAGGGCAACAAC |
|        |          | Rev- GTTCATGGGCTGTCGAGGC  |
| 2      | -481 to -244 | For- GTTACTTAGCTGAAGGGCACC|
|        |          | Rev- GGCCGGTTCTAAACTTCAAATG |
| 3      | -1048 to -655 | For- CAGTCATCAGGACTTGTGGT|
|        |          | Rev- CTGACGTCAGTATCAGGCC  |
| 4      | -1896 to -1554 | For- GGTATTCCCGCATTTACGTGTC|
|        |          | Rev- CACTGATTCCCCCAGCAAC  |
Figure 1
Figure 2
Figure 3
Figure 4
Extracellular UDP-Glucose Activates P2Y14 Receptor, Induces Signal Transducer and Activator of Transcription 3 (STAT3) T705 Phosphorylation and Binding to Hyaluronan Synthase 2 (HAS2) Promoter, Stimulating Hyaluronan Synthesis of Keratinocytes

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