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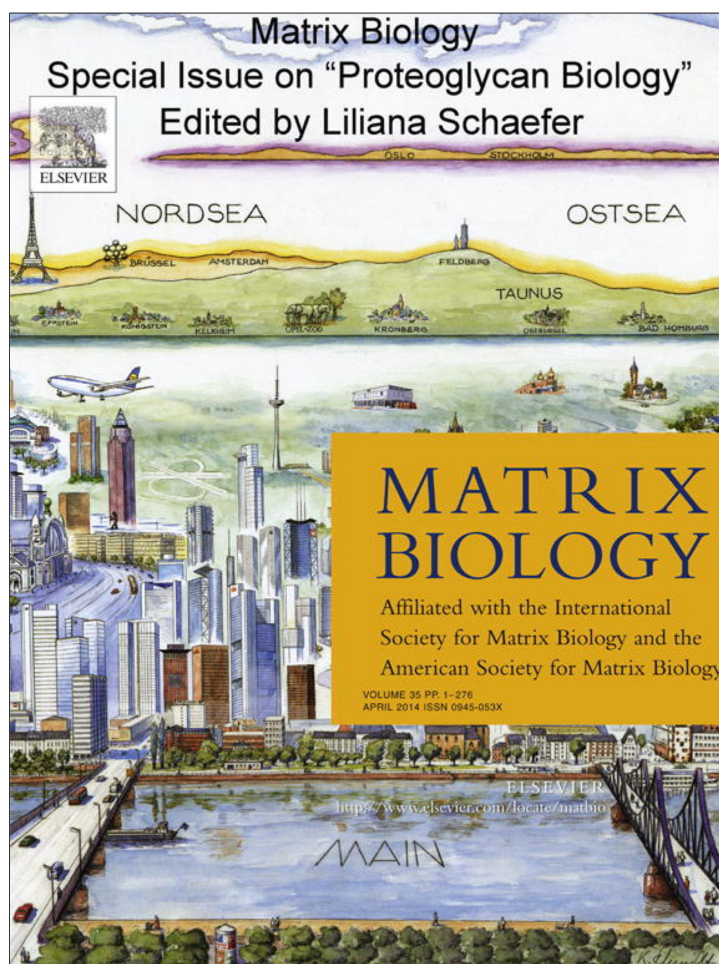

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Matrix Biology 35 (2014) 8–13

Contents lists available at ScienceDirect

Matrix Biology

journal homepage: www.elsevier.com/locate/matbio

Metabolic control of hyaluronan synthases

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ARTICLE INFO

Available online 14 October 2013

Keywords:
Glycosaminoglycan
UDP-GlcUA
UDP-GlcNAc
AMPK
O-GlcNAcylation
HBP

ABSTRACT

Hyaluronan (HA) is a glycosaminoglycan composed by repeating units of D-glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) that is ubiquitously present in the extracellular matrix (ECM) where it has a critical role in the physiology and pathology of several mammalian tissues. HA represents a perfect environment in which cells can migrate and proliferate. Moreover, several receptors can interact with HA at cellular level triggering multiple signal transduction responses. The control of the HA synthesis is therefore critical in ECM assembly and cell biology; in this review we address the metabolic regulation of HA synthesis. In contrast with other glycosaminoglycans, which are synthesized in the Golgi apparatus, HA is produced at the plasma membrane by HA synthases (HAS1-3), which use cytoplasmic UDP-glucuronic acid and UDP-N-acetylglucosamine as substrates. UDP-GlcUA and UDP-hexosamine availability is critical for the synthesis of GAGs, which is an energy consuming process. AMP activated protein kinase (AMPK), which is considered a sensor of the energy status of the cell and is activated by low ATP:AMP ratio, leads to the inhibition of HA secretion by HAS2 phosphorylation at threonine 110. However, the most general sensor of cellular nutritional status is the hexosamine biosynthetic pathway that brings to the formation of UDP-GlcNAc and intracellular protein glycosylation by *O*-linked attachment of the monosaccharide β-N-acetylglucosamine (O-GlcNAcylation) to specific aminoacid residues. Such highly dynamic and ubiquitous protein modification affects serine 221 residue of HAS2 that lead to a dramatic stabilization of the enzyme in the membranes.

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1. Introduction

The complex molecular structure that surrounds cells in tissues is defined as extracellular matrix (ECM) which has been reported to have many critical functions in determining the cellular microenvironment. A large body of evidence supports the concept that ECM not only acts in the structural architecture of the cells and tissues, but also regulates cell behavior in both physiological and pathological conditions controlling nutrient and waste exchanges, cell–cell and cell–matrix interactions, and diffusion of signaling molecules. Nowadays, the active role of ECM in cell physiology and pathology determination, for instance, proliferation, movements and differentiation of the cells as well as the stem cell niche is becoming more evident. ECM is also critical in cell dysfunctions that characterize many pathologies as cancer or cardiovascular diseases (Vigetti et al., 2006a; Akita et al., 2008; Acloque et al., 2009).

Among the different components of ECM, polysaccharides (i.e.,glycosaminoglycans, GAGs) have the highest variability and represent the most dynamic structures in tissues. In fact, several chemical modifications as epimerization, acetylation, O-, and N-sulfation are critical to determine the properties of chondroitins, heparan sulfate, and keratan. A plethora of enzymes are known to tailor GAG molecules during pathophysiological processes (Afratis et al., 2012). A further degree of variability is represented by the proteoglycan (PG) core protein, to which the GAG chains are linked. In fact, PGs can have several isoforms and can be differently modified by proteases and other post-translational modifications. Hyaluronan (HA) is an atypical GAG being not covalently bond to any PG core proteins, not epimerizated and not sulfated, although tumor necrosis factor alpha-stimulated protein 6 and its covalent binding to HA can mediate the transfer of the heavy chain of inter alpha trypsin inhibitor to −COOH of HA leading to a complex known as Serum-derived Hyaluronan-associated Protein (SHAP), that has proinflammatory properties and is involved in many pathologies (Jiang et al., 2011).

HA is simply composed by the disaccharide unit D-glucuronic acid (GlcUA) and D-N-acetyl-glucosamine (GlcNAc) linked by alternating β-1,4 and β-1,3 glycosidic bonds. HA is considered a molecule rich in biological information being the polymer length critical to determine HA effects (Stern et al., 2006). In fact, the number of HA disaccharide repetitions can greatly vary in determining the polymer molecular mass that ranges from 5 to 10,000 kDa. Further, high molecular weight HA can be fragmented in short oligosaccharides by different mechanisms including degrading enzymes (i.e., hyaluronidases), and free radicals (Stern et al., 2006). Moreover, low molecular mass HA can be directly synthesized by dysregulation of HA synthases (HASes) as well as due to an alter precursor availability. Therefore, the rheostatic effects of HA largely depend on the size of the polymeric chain. Further, several proteins with receptor functions have been described to bind HA directly affecting cell behavior (Jiang et al., 2011; Vigetti et al., 2011b). In

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http://dx.doi.org/10.1016/j.matbio.2013.10.002

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the light of these premises, HA cannot be considered a merely space filling molecule but a ubiquitous microenvironment component able to trigger different cell signaling cascades and to modulate many physiological and pathological processes. This review focuses on the fine mechanism that links cellular metabolism and HA synthesis also describing the known post-translational modifications of HASes in mammals.

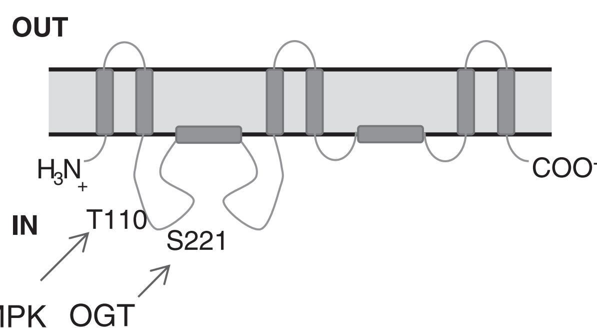
1.1. HA synthases

In mammals HA is synthesized by three different isoenzymes named HAS 1, 2, and 3. HASes are plasma membrane proteins that use cytoplasmic UDP-GlcNAc and UDP-GlcUA as precursors to polymerize the HA chain and to extrude it out of the cell without the necessity of a primer or an anchor protein or lipid. However purification experiments revealed that bacterial HAS (which shares several structural similarities with mammalian enzymes (Weigel and DeAngelis, 2007)) is strictly associated with cardiolipin and the catalytic activity is dependent on such lipid (Weigel et al., 1997). Although the crystallographic data are not yet available, HASes would have a complex architecture. In fact, a single protein codes for many functions as binding of two distinct UDP-sugars and binding of two distinct HA acceptor or donor species. Moreover HASes transfer two different sugars in two different linkages, catalyze repetitive sugar polymerization, and transfer HA across the membrane (Weigel and DeAngelis, 2007; Hubbard et al., 2012).

Little is known about HASes structure, topological *in silico* analyses revealed that Class 1 mammalian and Streptococcal HASes have 2 N-terminal transmembrane domains and several of C-terminal transmembrane domains or membrane associated domains (Jiang et al., 2011). In Fig. 1 are reported the predicted structural organization of mammals (Fig. 1A) and Streptococcal HASes (Fig. 1B). These domains are responsible for the catalytic activities of these proteins (Heldermon et al., 2001). On the other hand, the only member of Class 2 HAS, expressed by *Pasteurella multocida*, has a shorter C-terminal domain with only 4 transmembrane regions and 2 membrane associated domains. Interestingly, only a limited number of pathogenic bacteria show the ability to produce HA, which is used by these bacteria to shield themselves from the host immune system (DeAngelis, 1999). The cytosolic domain shares similarities with chitin synthases highlighting the importance of this portion of the protein for the catalysis. The overexpression of HAS2 and 3 lead to the formation of hetero and homodimers in Chinese hamster ovary (CHO) cells (Karousou et al., 2010) and this observation is consistent with the evidence that HAS activity resides in a large protein complex in plasma membranes (Asplund et al., 1998).

HASes possess different biochemical properties (Itano and Kimata, 2002). HAS1 is less catalytically active than HAS2, which, in turn, is intrinsically less active than HAS3. Moreover, HAS1 and HAS2 synthesize a polymer of high molecular weight whereas HAS3 produces shorter chains (~2×10^6 Da *vs* ~2×10^5 Da, respectively) (Itano et al., 1999). Interestingly, the lower intrinsic activity of HAS1 comes from the fact that it cannot produce HA when the cellular level of UDP-sugars is low (Rilla et al., 2013).

Many growth factors, cytokines, and other signaling molecules control the transcription of HAS genes and are recently reviewed elsewhere (Tammi et al., 2011). Once transcribed, to reach the plasma membrane, HAS proteins have to be synthesized in the endoplasmic reticulum and to follow the secretory pathway. Interestingly, microsomal membrane preparations, without plasma membrane contaminations, showed the capacity to synthesize HA in vitro. These properties are evident after several cell treatments that modulate ER stress, protein N-glycosylation and phosphorylation (Vigetti et al., 2009a). These latter evidences suggest that HASes enzymes, once translated, are subjected to a fine regulation that is largely unknown, nevertheless can be reasonable to hypothesize that modifications of



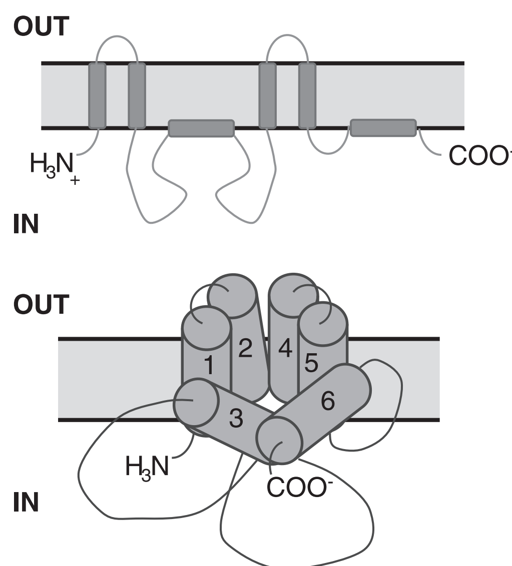


Fig. 1. A. Schematic representation of Class 1 mammal HAS with 6 transmembrane domains and 2 membrane associated domains. B. Schematic representation of Streptococcal Class 1 HAS with 4 transmembrane domains and 2 membrane associated domains (B, upper panel). In the lower panel there is a representation of Class 1 HAS in which the two cytoplasmic loops and the central pore for HA chain extrusion in the extracellular space are highlighted.

HASes can have a critical role in HASes activity. Moreover, evidences *in vitro* on purified bacterial (*P. multocida*) HAS showed that a monodispersed HA chain can be obtained by finely tuning the reaction stoichiometry. In fact the molar ratio of precursors and acceptor molecules has an important role in enzyme kinetics (Jing and DeAngelis, 2004; DeAngelis, 2008). This point highlights the crucial role of UDP-sugar precursors and, therefore, of the entire cell metabolism that produces HAS substrates.

2. Role of UDP-GlcUA and cellular energy homeostasis

UDP-GlcUA has many functions in mammalian cells in addition to being a component of the GAGs with the exception of keratan sulfate. In fact, UDP-GlcUA is the donor substrate for glucuronidation reactions that take place in liver to detoxify the organism and in other cell types (Vigetti et al., 2009b). Moreover, UDP-GlcUA can be decarboxylated to UDP-xylose which is critical for the linking of GAGs to proteoglycan core proteins.

To investigate the role of UDP-GlcUA in GAG synthesis, our group overexpressed UDP-glucose dehydrogenase, the enzymes involved in the oxidation of UDP-glucose to form UDP-GlcUA (Fig. 2), in human

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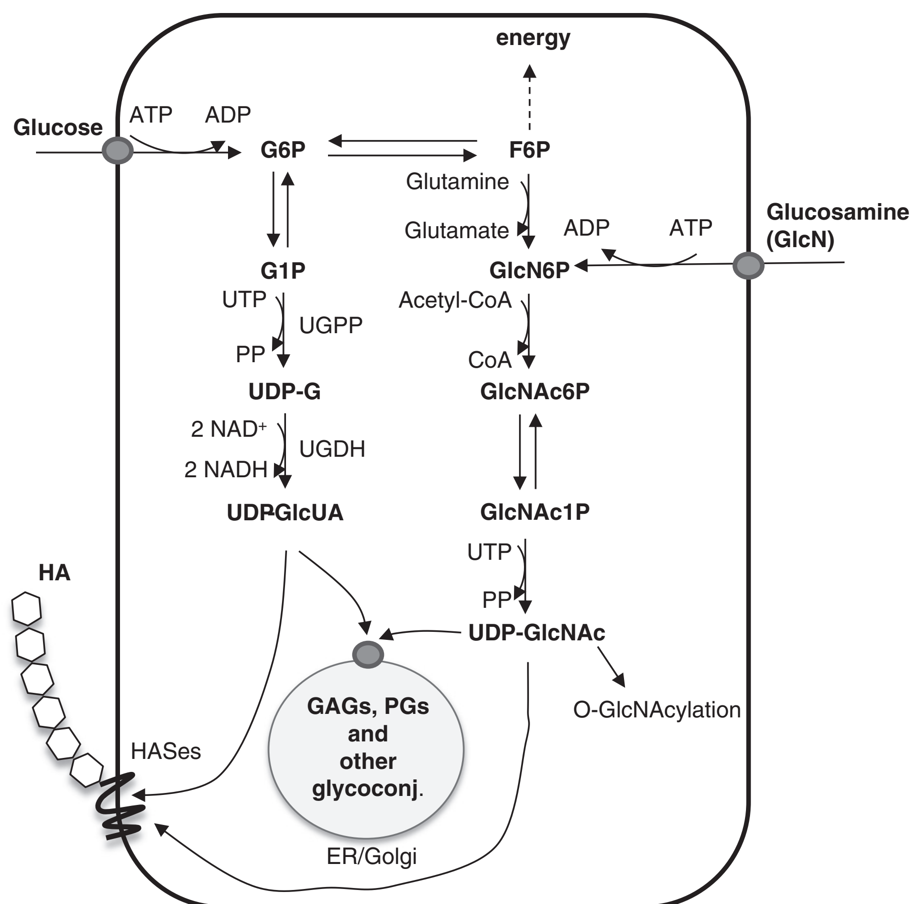

Fig. 2. Schematic representation of the UDP-GlcUA biosynthetic pathway and the hexosamine biosynthetic pathway. Glucose and glucosamine (GlcN) enter the cell through the GLUT transporters. UDP-glucose pyrophosphorylase (UGPP) and dehydrogenase (UGDH) bring to the formation of UDP-glucose (UDP-G) and UDP-GlcUA, respectively, starting from glucose 1-phosphate. UDP-GlcUA and UDP-GlcNAc can be directly used for the synthesis of HA by HAS enzymes located in the plasma membrane or can be transported inside the ER/Golgi for the synthesis of other glycoconjugates, especially proteoglycans.

cells and found an increment in the production of HA without altering the other GAG synthesis (Vigetti et al., 2006b). This point is of crucial importance because it could be an evidence that the UDP-sugar concentrations can vary in the cytosol and not inside the Golgi, where the sulfated GAGs are synthesized. The high affinity transporters of sugar nucleotides located on the Golgi membranes (Berninsone et al., 2001; Hoflich et al., 2004) are critical in maintaining a high concentration of UDP-sugars inside the Golgi, even if the cytosolic availability of such precursors is low or limiting. In this condition the only GAG to be affected by UDP sugar availability is HA, as HASes use precursors directly from the cytosolic pools. On the other hand, an increase of cytosolic UDP-sugars should have a minor impact on GAG synthesis in the Golgi as the saturation of the transporters, but have a significant effect on HA production. Therefore HA is the only GAG to be directly affected by UDP-sugar metabolism. Interestingly, an increase of UDP-glucose dehydrogenase (UGDH) was described during aging and linked with HA accumulation and signaling alteration (Vigetti et al., 2008b; Simpson et al., 2010; Cargill et al., 2012).

Other experiments in chondrocytes showed that also UDP-glucose pyrophosphorylase (UPP), the enzyme that catalyzes the formation of UDP-glucose from glucose-1 phosphate and UTP (Fig. 2) is critical for HA synthesis. In fact, the overexpression of UPP leads to an increase of HA accumulation (Magee et al., 2001). Besides its role in GAG synthesis, UDP-glucose is a critical substrate for glycogen synthesis. On this basis, it is clear that the fate of UDP-glucose has to be finely tuned, in fact all the reactions that involve UDP-glucose are essentially irreversible in all cellular conditions. As glycogen is a key component for cellular energy homeostasis, our hypothesis is that the energy charge (i.e., ATP:AMP ratio) can regulate UDP-GlcUA availability and GAG synthesis.

The main sensor of the energy status of the cell is represented by AMP activated protein kinase (AMPK) (Hardie, 2004). AMPK is a heterotrimeric cytosolic enzyme consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). This kinase is allosterically activated by an increase of the AMP:ATP ratio (Towler and Hardie, 2007), which occurs under conditions of cellular stress or energy deficiency, such as hypoxia, ischemia, and glucose deprivation (Towler and Hardie, 2007). AMPK is also activated through the phosphorylation of a conserved threonine in its activation loop (threonine 172) by upstream kinases as LKB1 (Hardie et al., 2006). When the ATP:AMP ratio decreases, AMPK inhibits anabolic processes and induces catabolic pathways in order to restore ATP levels (Towler and Hardie, 2007). By altering gene expression or by direct phosphorylation of several pivotal enzymes, AMPK regulates different cellular metabolic pathways, including glycolysis, gluconeogenesis, lipid metabolism, and protein synthesis.

GAG synthesis needs energy not just in terms of ATP, but also because it requires molecules involved in energy production as acetyl-CoA. Moreover, other critical molecules in cell metabolism are necessary for GAG synthesis, as glutamine, PAPS, and UTP, all depending on several metabolisms which regulate the synthetic pathway of sugars, amino acids and nucleotides. Moreover, the synthesis of UDP-GlcUA, producing two NADH molecules, can interfere with the NAD^+:NADH ratio, linking glucuronic acid synthesis with cell reducing potential. Interestingly, AMPK inhibited HA synthesis without altering the sulfated GAG production in human aortic smooth muscle cells (Vigetti

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et al., 2011a). Moreover, in human dermal fibroblast, the chemical activation of AMPK with AICAR, which mimics AMP inside the cells, slightly induces HAS2 messenger accumulation (Yamane et al., 2011) suggesting a complex and cell specific regulation of HAS2 transcription or HAS2 mRNA stability.

It was suggested that HASes activity is phospholipid-dependent (Weigel and DeAngelis, 2007) and AMPK can regulate the lipid microenvironment around HAS enzyme and, indirectly, modulate HAS activity or stability. We found that HAS2 enzymatic activity is dramatically inhibited after AMPK activation and this was due to the phosphorylation of threonine 110 of HAS2 (Vigetti et al., 2011a). As this residue is a conserved consensus for AMPK and the threonine 110 is located in the cytoplasmic loop of HAS2, this finding leads to the possibility that AMPK directly can modify HAS2 whereas the other enzymes for GAGs synthesis, located in the Golgi and not accessible to AMPK, are unaffected by AMPK. This aspect is confirmed by experiments using the AMPK activators, as AICAR, metformin or 2-deoxyglucose, which influenced only HA synthesis and not sulfated GAG production (Vigetti et al., 2011a). Interestingly the levels of mRNAs coding for the three HASes were not altered by AMPK (Vigetti et al., 2011a).

A previous study reported the phosphorylation of HAS3 without any effect on enzyme functionality (Goentzel et al., 2006), whereas another work clearly showed that ERK-mediated serine phosphorylation of all the three HASes increased their specific activity (Bourguignon et al., 2007). Interestingly, the treatments of plasma membranes or microsomal membranes with phosphatases or N-glycosidase F revealed that also HAS activity in internal vesicles can be modulated (Vigetti et al., 2009a). These findings suggest that HAS proteins have different and complex regulatory mechanisms in which several post-translational modifications in different sites can increase or decrease the enzymatic activity also during the secretory pathway. Moreover, a further level of regulation can derive from the observation that HAS2 and 3 can make homo and heterodimers, and that HAS2 activity can be modulated by ubiquitination at Lys190 (Karousou et al., 2010). Such results suggest that HASes, and HAS2 in particular, can be complex proteins with several possibilities for post-translational modifications capable of modulating enzymatic activity.

These observations suggest that the covalent modifications of the enzymes can be obtained very fast, and the cell reaction to the stimuli can take place in minutes. This concept introduces the idea that HASes can be considered “ready to use” enzymes, that means that their fast turnover and their presence in microsomal domain allow the cells to modulate very efficiently and rapidly the HA synthesis.

3. Role of UDP-GlcNAc and protein O-GlcNAcylation

Little is known about the availability of UDP-GlcUA and UDP-glucose when AMPK is activated, as in the case of nutrient shortage. In smooth muscle cells, UGPP and UGDH expressions were not modified after treatments with AICAR and metformin (Vigetti et al., 2011a). This finding can be explained considering that UDP-glucose and UDP-GlcUA are critical not only for HA synthesis, but also for all other sulfated GAGs, glycogen and other glycoconjugates that are all necessary for cell survival. Noteworthy, a proteomic study revealed that UGPP can be a target of AMPK in pigs, enhancing the level of complexity of this issue (Hedegaard et al., 2004).

The other critical sugar nucleotide for the synthesis of GAGs is UDP-GlcNAc (Fig. 2) which can be easily converted to UDP-GlcNAc by UDP-galactose 4-epimerase (Thoden et al., 2001). UDP-GlcNAc is one of the most abundant UDP-sugars within the cell (ranging from 100 μM to low millimolar, values comparable to ATP) and it is known that the UDP-GlcNAc concentration can greatly fluctuate (Tomiya et al., 2001; Marshall et al., 2004). Such variability depends not only on the amount of glucose that enters in the hexosamine biosynthetic pathway (HBP) (typically 2–5%) (McClain and Crook, 1996), but also depends on other pathways which regulate fatty acid, nucleotide and aminoacid metabolisms (Hanover et al., 2012). In fact, the hexosamine biosynthetic pathway integrates the nutrient status of the cell by utilizing glucose, acetyl-CoA, glutamine, and UTP to produce UDP-GlcNAc which can be considered an efficient nutrient sensor (Fig. 3).

In addition to glycoconjugates, UDP-GlcNAc can be used to glycosylate nucleo- and cytoplasmic proteins with β-*O*-linked GlcNAc (O-GlcNAcylation) (Butkinaree et al., 2010). O-GlcNAcylation is a reversible post-translational modification of serine/threonine that often alternates or competes with protein phosphorylation and is controlled by two enzymes: O-GlcNAc transferase (OGT) and O-GlcNAc hydrolase (OGA) (Hart et al., 2007; Hart et al., 2011) (Fig. 3). O-GlcNAcylation regulates many cellular functions including signaling, gene expression, degradation, and trafficking (Hart et al., 2007) and, interestingly, appears to be particularly sensitive to physiological flux of the UDP-GlcNAc pools (Love et al., 2010). Interestingly, a recent paper by Rilla and collaborators demonstrates the large range of UDP-sugar contents presented by different cell types, and showed a correlation between the expression of different HASes and the UDP-sugars (Rilla et al., 2013).

Increments of UDP-GlcNAc due to glucosamine or glucose uptake greatly induced the accumulation of GAGs (HA and chondroitins) in the medium of human smooth muscle cells (Vigetti et al., 2012). Such GAG increase is also permitted by a concomitant augment of UDP-GlcUA with a not yet known mechanism (Jokela et al., 2011). Interestingly the increment of O-GlcNAcylation without altering UDP-GlcNAc concentration (i.e., by inhibiting OGA with O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAC)) significantly induced only in HA production (Vigetti et al., 2012). This specific effect on HA can be ascribed to the O-GlcNAcylation of serine 221 of HAS2 which greatly stabilizes the enzyme in the membrane. HAS2 has a very rapid turnover with a calculated half-life of 17 min due to 26S proteasomal degradation (Vigetti et al., 2012). This instability, together with a very low level of HAS2 transcript, contributes to render HAS2 a very low expressed enzyme in cells. O-GlcNAcylation of HAS2 in the serine 221 is located in the cytosolic loop, does not alter the enzymic activity and increases the enzyme half-life to more than 5 h, permitting an increased HA synthesis (Vigetti et al., 2012).

The mechanism of HAS2 proteasomal degradation is complex and requires additional processes considering the several transmembrane

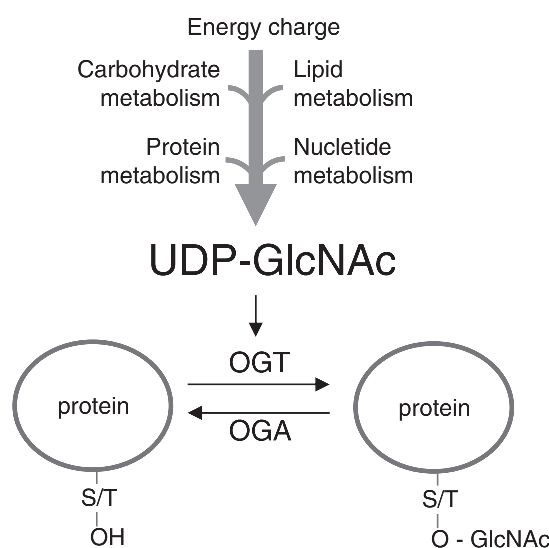

Fig. 3. Schematic representation of protein O-GlcNAcylation. UDP-GlcNAc concentration in cell cytosol depends on several pathways and is critical in regulating OGT activity. OGT catalyzes the transfer of the GlcNAc moiety from UDP-GlcNAc to the hydroxyl group of threonine or serine. OGA hydrolyzes the GlcNAc from the O-GlcNAcylation to form unmodified proteins.

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domains of HAS2 and its localization in the plasma membrane. Other mechanisms can be encountered considering the presence of active HAS2 in intracellular vesicles that can be part of the secretory pathway, or the possible internalization of the enzyme by endocytosis or plasma membrane recycling (Vigetti et al., 2009a). Several plasma membrane proteins are known to be ubiquitinated and degraded by the proteasome (Sepp-Lorenzino et al., 1995; Jeffers et al., 1997), whereas other plasma membrane proteins were internalized by endocytosis, and degraded by lysosomes (Bonifacino and Weissman, 1998). HAS2 is known to be poly- and mono-ubiquitinated and, while monoubiquitination seems to be involved in enzyme dimerization, polyubiquitination does not seem directly related to proteasomal degradation (Karousou et al., 2010). Recently, it was described that OGA can promote the degradation of surface lipid droplet proteins via the proteasome (Keembiyehetty et al., 2012) and, therefore, it can have a role in the regulation of HAS2 stability. Moreover, OGT is also present in the endoplasmic reticulum (ER) (Sakaidani et al., 2012), which could regulate the HAS2 folding process or its escape from the ER. Proteasomal degradation is typically associated with protein misfolding events within the ER, and this could also be involved in the regulation of HAS2. ER stress is known to modulate HA synthesis by altering HAS activity (Vigetti et al., 2009a). Finally, the process to deliver HAS2 to the proteasome is a complex mechanism and occurs in different cell conditions. Based on these considerations, to shed light on this issue additional studies are required, addressing specifically the HASes traveling throughout the cells and their covalent modifications and catalytic activities.

From a pathological point of view, the regulation of HAS2 by O-GlcNAcylation can have important therapeutic consequences considering that the excess of glucose can lead to a dramatic increase of UDP-GlcNAc and HA (in particular in cells where the uptake of glucose is insulin independent). Clinical and experimental evidences show that in hyperglycemic patients and in streptozotocin-induced diabetes animals there is evidence of HA accumulation both in plasma and in vascular wall (Heickendorff et al., 1994; McDonald et al., 2007). Since HA has pro-atherosclerotic properties (Vigetti et al., 2008a), stimulating cellular migration, and proliferation and modulating inflammation, it could be linked with macro- and microvascular diseases that represent a common complication in diabetic patients.

4. Conclusions

GAGs are a critical component of cellular microenvironment and are able to modulate cell behavior directly by binding to specific receptors/proteins or indirectly by sequestering growth factors and other signaling molecules. As HA is ubiquitous in the body, it is involved in many physiological and pathological processes. Recently, several evidences described a critical role of HA in inflammation, shedding light on this GAG role in human pathology and revealing its pivotal role in modulating immune cell differentiation, adhesion and activation. A deeper understanding of the specific mechanisms that regulate HA synthesis *in vivo* represents a new pharmacological target to treat pathologies in which the increase (i.e. cancer, diabetes) or decrease (joint disorders) of HA synthesis can have effects of clinical relevance.

Acknowledgments

The studies here described made by the A.P. group were supported by Fondazione Comunitaria del Varesotto-ONLUS, FAR and Centro Insubre di Biotecnologie per la Salute Umana (young researcher award to D.V.) and by the FP7 EU project "INFLAMA" to A.P. The authors acknowledge the "Centro Grandi Attrezzature per la Ricerca Biomedica", Università degli Studi dell'Insubria, for instrument availability.

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