Tlapak-Simmons et al. J Glycom Lipidom 2011, S:1
DOI: 10.4172/2153-0637.S1-001

Journal of Glycomics & Lipidomics

Research Article Open Access

Clustered Conserved Cysteines in Hyaluronan Synthase Mediate Cooperative Activation by Mg^{2+} Ions and Severe Inhibitory Effects of Divalent Cations

Valarie L. Tlapak-Simmons, Andria P. Medina, Bruce A. Baggenstoss, Long Nguyen, Christina A. Baron and Paul H. Weigel*

Department of Biochemistry & Molecular Biology, The Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA

Abstract

Hyaluronan synthase (HAS) uses UDP-GlcUA and UDP-GlcNAc to make hyaluronan (HA). *Streptococcus equisimilis* HAS (SeHAS) contains four conserved cysteines clustered near the membrane, and requires phospholipids and Mg^{2+} for activity. Activity of membrane-bound or purified enzyme displayed a sigmoidal saturation profile for Mg^{2+} with a Hill coefficient of 2. To assess if Cys residues are important for cooperativity we examined the Mg^{2+} dependence of mutants with various combinations of Cys-to-Ala mutations. All Cys-mutants lost the cooperative response to Mg^{2+}. In the presence of Mg^{2+}, other divalent cations inhibited SeHAS with different potencies (Cu^{2+}~Zn^{2+} >Co^{2+} >Ni^{2+} >Mn^{2+} >Ba^{2+} Sr^{2+} Ca^{2+}). Some divalent metal ions likely inhibit by displacement of Mg^{2+}-UDP-Sugar complexes (e.g. Ca^{2+}, Sr^{2+} and Ba^{2+} had apparent K_i values of 2-5 mM). In contrast, Zn^{2+} and Cu^{2+} inhibited more potently (apparent $K_i \leq 0.2$ mM). Inhibition of Cys-null SeHAS by Cu^{2+}, but not Zn^{2+}, was greatly attenuated compared to wildtype. Double and triple Cys-mutants showed differing sensitivities to Zn^{2+} or Cu^{2+}. Wildtype SeHAS allowed to make HA prior to exposure to Zn^{2+} or Cu^{2+} was protected from inhibition, indicating that access of metal ions to sensitive functional groups was hindered in processively acting HA●HAS complexes. We conclude that clustered Cys residues mediate cooperative interactions with Mg^{2+} and that transition metal ions inhibit SeHAS very potently by interacting with one or more of these –SH groups.

Keywords: Streptococcal; Enzyme kinetics; Cooperativity; Mutagenesis; Cysteine cluster

Abbreviations: CL: Cardiolipin; HA: Hyaluronic Acid, Hyaluronate, Hyaluronan; HAS: HA Synthase; SeHAS: *Streptococcus equisimilis* HAS; SpHAS: *Streptococcus pyogenes* HAS

The linear glycosaminoglycan hyaluronan (HA) is composed of unmodified disaccharide repeats: [-D-glucuronic acid-(β1,3)-D-N-acetylglucosamine-(β-1,4)]$_n$. HA is ubiquitous in vertebrate extracellular matrices, and it is a major component in tissues such as cartilage and dermis, and in synovial and vitreous fluids [1,2]. HA is also made by some prokaryotes and fungi [3-5]. HA plays an important role during fertilization, embryogenesis, development, wound healing, and differentiation [6,7] and in a variety of cellular functions such as migration and matrix assembly [8-10].

The streptococcal and eukaryotic Class I HASs are membrane proteins [11] containing 6 or 8 membrane domains, respectively, with topologies predicted to be similar to that of SpHAS (Figure 1A), which was determined experimentally [12]. The mammalian enzymes belong to three subfamilies, designated HAS1, HAS2 and HAS3, which are ~30% identical to the streptococcal HASs from *pyogenes*, *equisimilis,* and *uberis*. The molecular masses of the streptococcal synthases are ~49 kDa, which is relatively small to mediate the multiple functions required for HA synthesis [13]. HAS enzymes must bind the UDP-GlcUA and UDP-GlcNAc precursors, catalyze two distinct glycosyltransferase reactions, bind to the growing HA-UDP chain, translocate the growing HA polymer through the enzyme and thus the cell membrane (Figure 1B), and finally release the HA chain to the cell surface or medium; typically after >10,000 disaccharide units (>4 x 10^6 Da) have been polymerized.

Cell-free HA biosynthesis was achieved in 1959 [3] and active HAS preparations were later obtained from detergent extracts of eukaryote [14,15] and prokaryote [16,17] membranes. Active purified recombinant enzymes were ultimately reported for Group A *Streptococcus pyogenes* HAS (SpHAS) and Group C *Streptococcus equisimilis* HAS (SeHAS) expressed in *E. coli* [18] and for mouse HAS1 expressed in COS-1 cells [19]. The streptococcal enzymes have been characterized kinetically [20,21], and shown to function as monomers [22] in complex with phospholipids [18], particularly cardiolipin (CL). The phosopholipid composition of *E. coli* membranes is typically (in mole %): 75% PG, 20% PE, and 5% CL. This CL content supports a high level of recombinant SeHAS expression (~10% of total membrane protein) with high activity [23]. Based on its' lipid-dependence, multiple membrane domains and processive mechanism, we proposed that SeHAS contains an intraprotein pore (Figure. 1B) through which HA is synthesized and simultaneously translocated across the membrane to the cell exterior [18]. The Class I HASs mediate HA chain growth at the reducing end [24-26]. In this case, the UDP-sugars are acceptors and hyaluronyl-UDP chains are the donors in the two glycosyltransferase reactions required to make HA [13].

SeHAS is the smallest (417 amino acids) member of the Class I HAS family [27] and contains only four Cys residues (at positions 226, 262, 281, and 367) that are highly conserved within the Class I HASs, in particular in all the mammalian HASs. We previously created a panel of Cys-to-Ala mutants in order to study the role of these residues in HAS structure and function [28,29]. We found that the four Cys residues in active enzyme are not disulfide bonded and they are not required for activity, although Cys modification by sulfhydryl reagents (e.g. N-ethylmaleimide) can severely inhibit the enzyme .

***Corresponding author:** Paul H. Weigel, Department of Biochemistry & Molecular Biology, The Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA, Tel: 405-271-1288; Fax 405-271-3092; E-mail: paul-weigel@ouhc.edu

Received August 19, 2011; **Accepted** November 11, 2011; **Published** November 15, 2011

Citation: Tlapak-Simmons VL, Medina AP, Baggenstoss BA, Nguyen L, Baron CA, et al. (2011) Clustered Conserved Cysteines in Hyaluronan Synthase Mediate Cooperative Activation by Mg^{2+} Ions and Severe Inhibitory Effects of Divalent Cations. J Glycom Lipidom S1:001. doi:10.4172/2153-0637.S1-001

Here we investigated the involvement of these four SeHAS Cys residues in the cooperative stimulation of enzyme activity by Mg^{2+}, and the sensitivity of HAS activity to other ions, including transition metal ions. We found that the enzyme response to Mg^{2+} was sigmoidal, rather than hyperbolic, and abrogated in all Cys-mutants. Cys residues are also responsible for most of the severe inhibition by Cu^{2+}, but not by Zn^{2+}. The results indicate important though subtle roles for the four conserved Cys residues in the HAS catalyzed assembly of HA.

Materials and Methods

Materials, strains and plasmids

Reagents were supplied by Sigma-Aldrich, unless noted otherwise. Media components were from Difco (Fisher Scientific). The gene encoding HAS from *S. equisimilis* was inserted into the pKK223-3 vector (Amersham Pharmacia Biotech) and cloned into *E. coli* SURE™ cells [27]. The gene in this vector is driven by the *tac* promoter, which is regulated by the *lac* repressor and induced with 1 mM isopropyl-β-D-thiogalactoside. The SeHAS gene contains a C-terminal in-frame sequence encoding a 6-His tail to facilitate enzyme purification [18].

Cell growth and membrane preparation: *E. coli* SURE™ cells containing SeHAS-encoding plasmids were grown at 30°C in Luria broth to an A_{600} ~1.6, induced for 3 hr, harvested and washed at 4°C, and stored frozen in PBS with 20% glycerol at -80°C. Membranes were prepared by osmotic shock, sonication and differential centrifugation as described previously [18]. Protein concentrations were determined with the Coomassie protein assay reagent (Pierce) using bovine serum albumin as the standard [30].

Preparation and characterization of SeHAS mutants: The generation of single, double, triple, and null Cys-to-Ala mutants of SeHAS by site directed mutagenesis, as well as their expression and kinetic characterization, were described previously [28]. All the mutants used here have substantial levels of activity, in some cases similar to wildtype.

HA synthase assays: The activity of wildtype and Cys-mutant SeHAS in membranes was determined in 100 µl of 25 mM Na_2KPO_4, pH 7.0, containing 50 mM NaCl, 20 mM $MgCl_2$ (unless indicated otherwise), 0.1 mM EDTA, 2 M glycerol, 1.0 mM UDP-GlcUA, 1.0 mM UDP-GlcNAc and 0.69 µM UDP-[^{14}C]GlcUA (380 mCi/mmol; New England Nuclear), a mixture of protease inhibitors, and the indicated divalent cation chloride salt to be tested. Membranes were added to initiate the enzyme reaction and the mixture was gently agitated in a MicroMixer E-36 (Taitec) at 30°C for 1 hr. For the conditions reported, the assays used limiting amounts of SeHAS protein and the kinetics of HA synthesis were linear. Reactions were terminated by the addition of SDS to a final concentration of 2% (w/v), and the incorporation of [^{14}C] GlcUA into HA was assessed by descending paper chromatography and scintillation counting as described previously [31]. HAS activity was normalized to total membrane protein as described previously [27,28]. Each datum point is the average of duplicates or triplicates, and the individual values were typically within 10%. Results with a particular combination of SeHAS mutant and divalent cation were verified in 2-3 independent experiments. For experiments to assess inhibition of purified enzyme by divalent cations, wildtype SeHAS was purified and assayed as described previously [21,18] in the presence of 20 mM $MgCl_2$, 2 mM bovine cardiolipin, and the indicated concentration of divalent cation chloride salt to be tested. The cooperativity of enzyme kinetc responses to increasing $MgCl_2$, concentration were assessed by calculating the Hill coefficient [32].

Results

The effect of Mg^{2+} concentration on SeHAS activity

The Mg^{2+} requirement for SpHAS activity has been known for over five decades [3], and all other HAS enzymes, such as SeHAS [27], XlHAS [33], and mammalian HASs [34] also require Mg^{2+}. This requirement for Mg^{2+} is considered to reflect the typical enzyme recognition of nucleotide-Mg^{2+} complexes. Surprisingly, the stimulation profile of membrane-bound wildtype SeHAS activity with increasing Mg^{2+} concentration was sigmoidal, with a Hill coefficient of 2, indicating a substantial cooperative binding behavior (Figures 2 and 3, open circles). Maximum stimulation of SeHAS activity occurred at 10-12 mM $MgCl_2$.

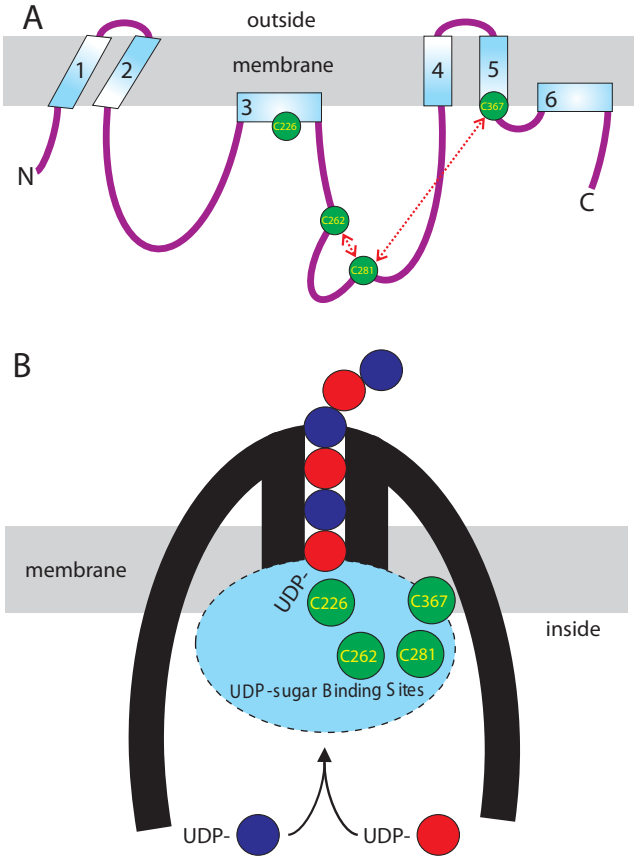

Figure 1: Conserved SeHAS Cys residues are clustered in the active sites and at the membrane surface. A. The linear scheme illustrates the topology and organization of HAS domains within the membrane and the relative locations of the four Cys residues, indicated in green circles, conserved in the Class I HAS family. C226 and C367 are located in membrane domains and are thus at the membrane interface. Based on chemical inhibition studies (Kumari and Weigel, 2005), C281 is in close proximity to C367 and C262 is close to C281, as indicated by the dashed red arrows. B. The compact scheme for organization of HAS (thick black lines) in the membrane illustrates the growing HA chain (alternating blue and red circles) being assembled by addition of the UDP-GlcUA and UDP-GlcNAc substrates at its reducing end, while it simultaneously is translocated through an intraprotein pore to the cell exterior. The four conserved Cys residues are localized at the membrane surface and are within the active sites (blue oval). Each catalytic cycle of the novel processive mechanism for HA biosynthesis by Class I HAS enzymes alternately adds HA-UDP to free UDP-GlcNAc or UDP-GlcUA to create HA with a new extended reducing end attached to UDP [13].

Citation: Tlapak-Simmons VL, Medina AP, Baggenstoss BA, Nguyen L, Baron CA, et al. (2011) Clustered Conserved Cysteines in Hyaluronan Synthase Mediate Cooperative Activation by Mg^{2+} Ions and Severe Inhibitory Effects of Divalent Cations. J Glycom Lipidom S1:001. doi:10.4172/2153-0637.S1-001

The cooperative response of membrane-bound SeHAS to Mg^{2+} is eliminated in single, double, triple or null Cys-mutants

To determine the possible involvement of Cys residues in the SeHAS response to Mg^{2+}, we assessed the Mg^{2+}-dependence of the single (Figure 2C), double or null (Figures 2A and 2B), and triple (Figures 3A and 3B) SeHAS Cys-mutants. The expression and activity characterization of these Cys-to-Ala mutants were previously reported [28].

The double Cys-mutants seemed most revealing, showing no sigmoidal activation in the presence of increasing Mg^{2+}, although at high concentrations all the mutants were substantially active (Figure 2A). In contrast, the sigmoidal behavior of wildtype SeHAS was very evident at lower $MgCl_2$ (Figure 2B). At concentrations ≥ 1 mM Mg2+,

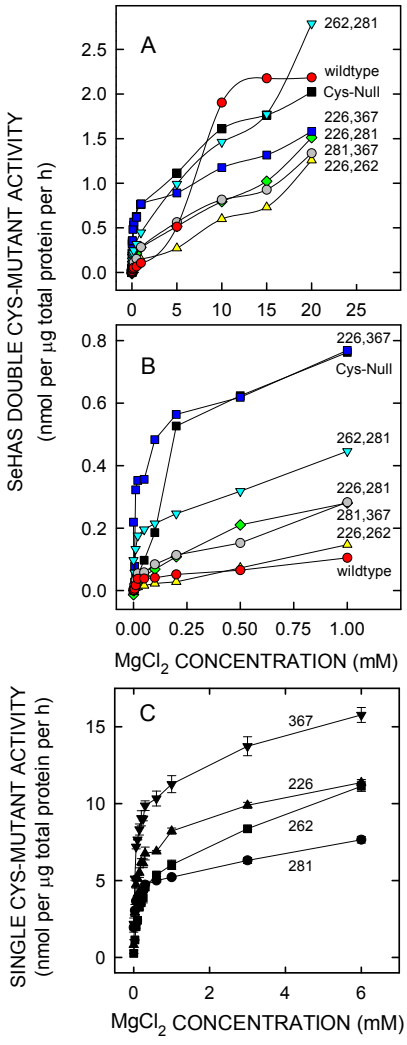

Figure 2: Effect of Mg^{+2} on the activity of SeHAS single, double, and null Cys-mutants. A. HAS activity was determined in the presence of increasing concentrations of $MgCl_2$, with no other divalent salts present, as described in Methods, using membranes containing wildtype or the indicated SeHAS double Cys-mutants. Panel B is a blow-up of the 0-1 mM range from A. The SeHAS variants are: wildtype, red circle; C(226,262)A, yellow triangle; C(226,281)A, green diamond; C(226,367)A, dark blue square; C(262,281)A, light blue inverted triangle; C(281,367)A, gray circle ; Cys-null, black square. C. Membranes containing single Cys-mutants were assayed as above: C(226)A, black triangle; C(262)A, black square; C(281)A, black circle; or C(367)A, black inverted triangle.

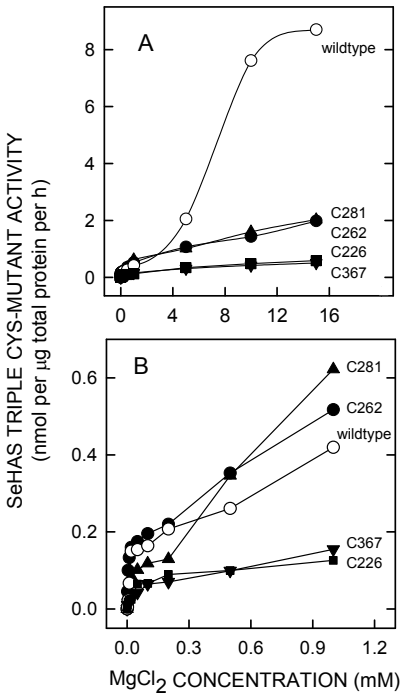

Figure 3: Effect of Mg^{+2} on the activity of SeHAS triple Cys-mutants. A. HAS activity was determined as in Figure 2 using membranes containing wildtype SeHAS or the four triple Cys-mutants. The single remaining Cys residue is designated for the indicated triple-Cys-mutants: wildtype, white circle; Δ3C(C226), ■; ; Δ3C(C262), ●; Δ3C(C281), ▲; Δ3C(C367), ▼. B shows the 0 - 1.0 mM region of panel A enlarged.

the mutants displayed several types of kinetic behavior. The null and C(226,367)A Cys-mutants, and to a lesser degree the C(262,281) mutant, were efficiently activated at ≤0.2 mM, and then showed nearly hyperbolic increases from 0.2-1.0 mM $MgCl_2$ (Figure 2B). In contrast, the C(226,281)A and C(281,367)A mutants were activated at low $MgCl_2$ just slightly more than wildtype. The C(226,262)A mutant and wildtype enzyme were virtually identical and were only minimally activated at low $MgCl_2$ (Figure 2B), consistent with a sigmoidal response. As the $MgCl_2$ concentration increased above 1 mM, all the mutants were stimulated in a nearly linear and parallel manner (Figure 2A). All the double Cys-mutants and the Cys-null enzyme were active at 15 mM $MgCl_2$ (35% to 85% of wildtype; Figure 2A). Wildtype enzyme activity was saturated at 15 mM $MgCl_2$, whereas activities of the mutants were still increasing. The results show that modifying any pair of Cys residues can drastically alter, but not eliminate, the response to increasing Mg^{2+} concentration.

Similarly, each of the four single Cys-mutants showed loss of the characteristic sigmoidal increase in enzyme activity with increasing $MgCl_2$ (Figure 2C). The initial enzymatic rates for all four mutants at low Mg^{2+} were faster than wildtype and essentially hyperbolic, although the activities of each mutant varied. Results with the four triple Cys-mutants were also similar in that none showed a cooperative response to increasing Mg^{2+} concentration (Figure 3). These mutants were substantially less active than wildtype enzyme (Figure 3A) and showed only modest hyperbolic profiles with increasing $MgCl_2$ concentration (Figure 3B).

Many divalent metal ions inhibit purified wildtype SeHA

The Mg^{2+} requirement for HAS activity has been known for decades, but had not yet been determined for a purified HAS enzyme.

Citation: Tlapak-Simmons VL, Medina AP, Baggenstoss BA, Nguyen L, Baron CA, et al. (2011) Clustered Conserved Cysteines in Hyaluronan Synthase Mediate Cooperative Activation by Mg^{2+} Ions and Severe Inhibitory Effects of Divalent Cations. J Glycom Lipidom S1:001. doi:10.4172/2153-0637.S1-001

As with the membrane-bound enzyme, the stimulation profile of SeHAS activity with increasing Mg^{2+} concentration was sigmoidal, indicating a cooperative binding behavior with a Hill coefficient of 2 (Figure. 4A; dashed line). Maximum stimulation of SeHAS activity occurred at 10 mM $MgCl_2$ with an apparent K_m of ~5 mM. The $MgCl_2$ concentration was then kept constant at 20 mM in order to examine the potential of other divalent metal ions to affect HA polymerization. An earlier report indicated that SpHAS has a small amount of activity with Mn^{2+} and Co^{2+} [3], but we found for SeHAS that none of the eight divalent metal ions tested (Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+} or Ca^{2+}) could effectively substitute for Mg^{2+} (not shown). Despite their inability to support HA synthesis, all of these divalent cations inhibited SeHAS activity in a dose-dependent manner (Figure 4A). Several transition metal ions, particularly Zn^{2+} and Cu^{2+} were inhibitory in the sub-mM range. The order of inhibitory effect (greatest-to-least) was: Cu^{2+}~ Zn^{2+}>Co^{2+} >Ni^{2+} >Mn^{2+} >Ba^{2+} >Sr^{2+} Ca^{2+}. The apparent K_i values for Zn^{2+} and Cu^{2+} were essentially identical at 100-200 μM, whereas the K_i for Ca^{2+} was ~12 mM. To assess the generality of the potent inhibition of SeHAS by Cu^{2+}, we also examined its effect on purified SpHAS, which contains six Cys residues - two others in addition to the four conserved Cys residues in SeHAS. Both enzymes showed virtually identical sensitivity to inhibition by $CuCl_2$ (Figure 4B).

Divalent cation inhibition of SeHAS Cys-mutants reveals multiple sensitivity components depending on the ion

The inhibition by Cu^{2+}, Zn^{2+} and the other ions could be due to a direct effect on the enzyme or to an indirect effect, in particular the displacement and substitution of Mg^{2+} ions in UDP-sugar complexes. To examine the potential involvement of the conserved Cys residues in the inhibition of SeHAS by the divalent cations noted above, we tested their effects on the activity of the membrane-bound Cys-mutants in the presence of 20 mM $MgCl_2$ (Figure 5). The double Cys and Cys-null mutants showed very different inhibition patterns with $CaCl_2$ (Figure 5A) and $CuCl_2$ (Figure 5B), chosen as the least and most potent inhibitors, respectively. The mutants and wildtype showed essentially the same sensitivities to Ca^{2+}, with most effects occurring between 0-1 mM and maximal inhibitions of 10-25% between 5-15 mM (Figure 5A). Thus, whether any Cys residues were present or not, all the variants retained roughly 80% of their activity in the presence of $CaCl_2$. In contrast, the double and null Cys-mutants displayed distinctly different inhibition patterns with $CuCl_2$ (Figure 5B), indicating that Cys residues are responsible for a substantial portion of the severe inhibition that essentially inactivates wildtype SeHAS. The C(226,367)A and C(281,367)A mutants were most similar to wildtype in their sensitivity to Cu^{2+}, whereas the C(262,281)A and Cys-null mutants were the most resistant to Cu^{2+}. The C(226,262)A and C(226,281)A mutants showed an intermediate sensitivity, retaining 20-40% of their activity between 1-10 mM $CuCl_2$ (while wildtype had <1% activity). Similar results were obtained with $CdCl_2$ (not shown).

Figure 5C shows the sensitivity of wildtype and Cys-null SeHAS to inhibition by <1 mM $CuCl_2$ or $ZnCl_2$. The Cys-null mutant retained 80% activity between 0.2-1.0 mM Cu^{2+}, whereas the wildtype enzyme was <1% active. In contrast, there was not much difference in relative resistance to $ZnCl_2$ between wildtype and Cys-null enzyme. These results indicate that the mechanisms by which Cu^{2+} and Zn^{2+} inhibit are different; that there are multiple mechanisms by which individual divalent cations can severely inhibit HAS. The sensitivity of the triple Cys-mutants to low concentrations (0-0.2 mM) of $CuCl_2$ illustrates this as well (Figure 6), since each mutant containing a different single Cys residue was much more active than wildtype enzyme, yet the mutants themselves showed different levels of activity (e.g. the mutant with C226 free was only 20% inhibited at 0.2 mM Cu^{2+}, while the mutant with C367 free was ~55% inhibited).

HA synthesis protects SeHAS from divalent metal ion inhibition

Inhibition by Zn^{2+} or Ca^{2+}, but not Cu^{2+}, was partially rescued by EGTA (not shown), indicating that the interactions between some metal ions and SeHAS may have a higher affinity and not be readily reversible. If Zn^{2+} or Cu^{2+} ions interact with the active sites of the enzyme, then SeHAS might be protected from inhibition if it was already catalytically engaged in HA biosynthesis. Protection by substrates is particularly likely for HAS, since it functions by a processive mechanism; HAS●HA-UDP complexes do not dissociate during biosynthesis, until the final product polymer is released [35,36]. To test this, the effects of the two ions were assessed after first incubating purified enzyme in the absence (no HA) or presence of UDP-sugars to allow HA synthesis for 5, 10 or 15 min (Figure 7). The subsequent sensitivity of SeHAS to inhibition by either Cu^{2+} (Figure 7A) or Zn^{2+} (Figure 7B) was substantially diminished, in a time-dependent way, by allowing the enzyme to make HA before exposure to these ions. After 15 min of HA synthesis, the levels of enzyme inhibition by Zn^{2+} and Cu^{2+} were similar to that of Ca^{2+} (Figures 4A and 5A), indicating a residual moderate effect on Mg^{2+}-UDP-sugar complex formation rather than an interaction with Cys residues. Thus, the inhibition by both Zn^{2+} and Cu^{2+} was substantially decreased when wildtype SeHAS was actively engaged in HA synthesis,

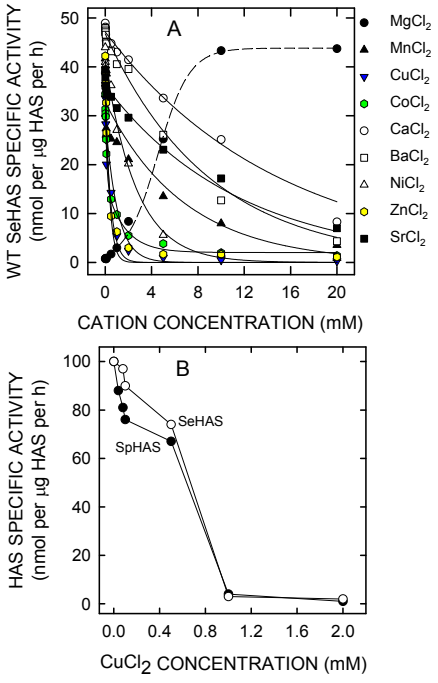

Figure 4: Mg^{2+}-dependence of purified SeHAS and the effects of other divalent cations on enzyme activity. A. Purified SeHAS was incubated with the indicated concentration of $MgCl_2$ in the absence of any other divalent metal ions (dashed line, black circles). In separate experiments, purified SeHAS in the presence of 20 mM $MgCl_2$ was incubated with the indicated concentrations of one of the eight divalent cation chloride salts noted in the symbol legend (right of figure), and HA synthase activity was quantified as described in Methods. The divalent cations salts tested were: $MnCl_2$ (black triangle); $CuCl_2$ (blue inverted triangle); $CoCl_2$ (green hexagon); $CaCl_2$ (white circle); $BaCl_2$ (white square); $NiCl_2$ (white triangle); $ZnCl_2$ (yellow hexagon); $SrCl_2$ (black square). B. Purified SeHAS (○) and SpHAS (●) were assayed in the presence of 20 mM $MgCl_2$ and the indicated concentration of $CuCl_2$.

Citation: Tlapak-Simmons VL, Medina AP, Baggenstoss BA, Nguyen L, Baron CA, et al. (2011) Clustered Conserved Cysteines in Hyaluronan Synthase Mediate Cooperative Activation by Mg^{2+} Ions and Severe Inhibitory Effects of Divalent Cations. J Glycom Lipidom S1:001. doi:10.4172/2153-0637.S1-001

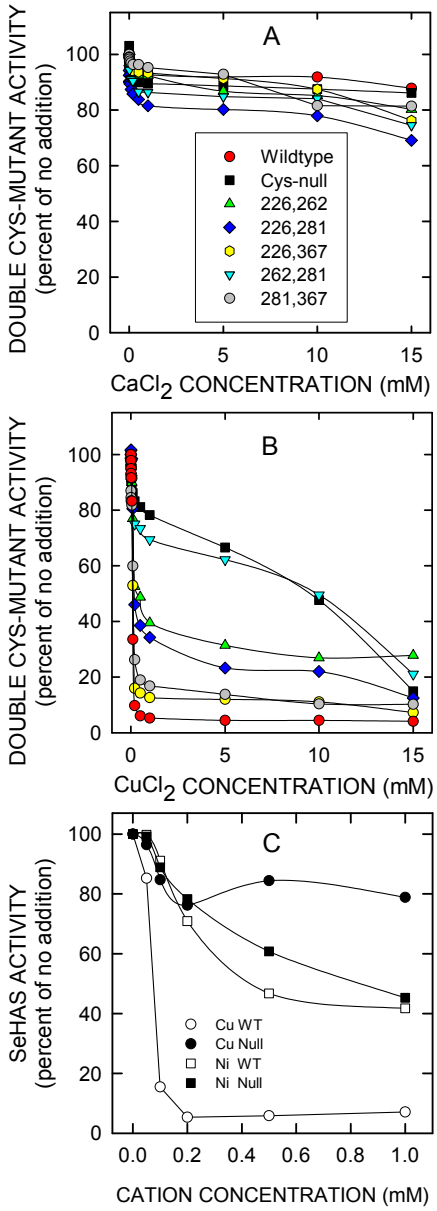

Figure 5: The activities of SeHAS wildtype, double and null Cys-mutants are differentially affected by Ca^{+2}, Ni^{+2} or Cu^{+2}. Membranes containing SeHAS wildtype, null, or double Cys-mutants (coded as noted in the Panel A insert) were incubated in the presence of 20 mM $MgCl_2$ and the indicated concentrations of either $CaCl_2$ (A) or $CuCl_2$ (B) and HA synthase activity was determined as described in Methods. The SeHAS variants in A and B are: wildtype, red circle; Cys-null, black square, C(226,262)A, green triangle; C(226,281)A, blue diamond; C(226,367)A, yellow hexagon; C(262,281)A, light blue inverted triangle; C(281,367)A, gray circle. C. Membranes containing wildtype (○, □) or Cys-null (●, ■) SeHAS were incubated in the presence of 20 mM $MgCl_2$ and the indicated concentrations of $CuCl_2$ (●, ○) or $NiCl_2$ (■, □) and HA synthase activity was determined as described in Methods.

consistent with hindered access to the clustered Cys residues in the active sites of HAS●HA-UDP complexes.

Discussion

An important point to note for the experiments performed in this study is that the *E. coli* SURE cells used for SeHAS expression do not support HA synthesis, since these cells lack the UDP-GlcUA precursor [31,37]. This was a key discovery that aided our initial successful cloning of SpHAS and, importantly, it means that the results presented here were obtained with membrane-bound or purified SeHAS that was

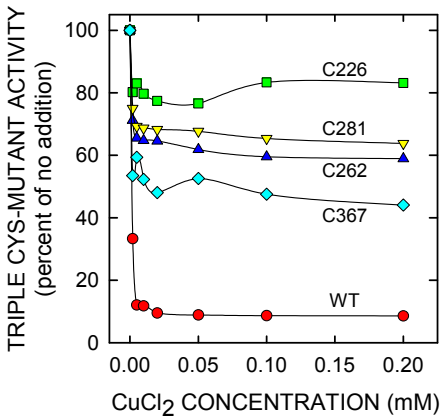

Figure 6: The activities of SeHAS triple Cys-mutants are differentially affected by Cu^{+2}. Membranes containing SeHAS wildtype or triple Cys-mutants were incubated in the presence of 20 mM $MgCl_2$ and the indicated concentrations of $CuCl_2$ and HA synthase activity was determined as described in Methods. The SeHAS variants (with the single free Cys indicated) were: wildtype, red circle; Δ3C(C226), green square; Δ3C(C262), dark blue triangle; Δ3C(C281), yellow inverted triangle; Δ3C(C367), light blue diamond.

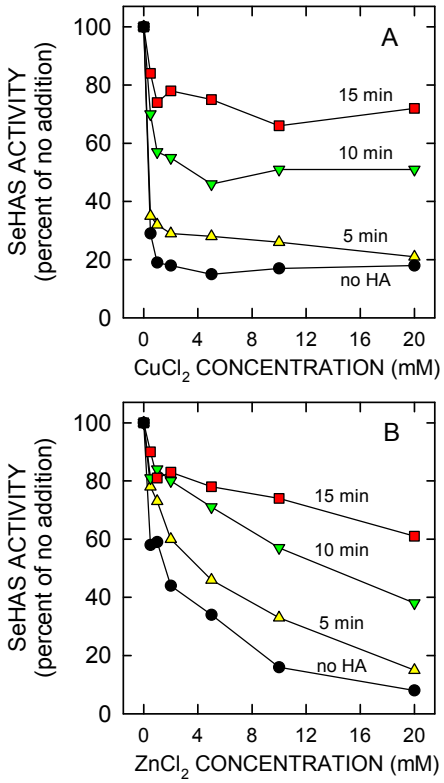

Figure 7: Purified SeHAS synthesizing HA is resistant to inhibition by $ZnCl_2$ or $CuCl_2$. SeHAS was purified as described in Methods and, in a pretreatment, samples were incubated without (no HA; black circles), or with UDP-sugars for 5 (yellow triangles), 10 (green inverted triangles) or 15 (red squares) min, after which the indicated concentration of $CuCl_2$ (A) or $ZnCl_2$ (B) was added and the samples were incubated an additional 30 min under normal assay conditions to assess relative enzyme activities.

free and not already in complex with HA-UDP (until UDP-sugars were added *in vitro*).

Many enzymes require divalent metal ions, with Mg^{2+} being one of the most prevalent, and SeHAS is no exception. Since glycosyltransferase substrates are UDP-sugars, it is expected that HASs utilize Mg^{2+} ions to help coordinate the pyrophosphoryl group of the substrates. Many X-ray structure examples are known in which Mg^{2+}, water, and amino acid side chains (e.g. carboxylates) interact to bind and orient the nucleotide in an active site, making it ready for catalysis [38]. The cooperative response to increasing Mg^{2+} concentration indicates that SeHAS contains multiple low affinity Mg^{2+} binding sites and that the initial binding of Mg^{2+} to the enzyme facilitates binding of additional Mg^{2+} ions. Interestingly, the original report of a Mg^{2+} requirement for SpHAS also showed a slight sigmoidal response [3]. A cooperative response for Mg^{2+} activation has not yet been tested for in any other purified Class I HAS.

We previously showed [23] that the four Cys residues in SeHAS are clustered together at the membrane-protein junction and are either part of, or very near, UDP-sugar binding sites (Figure 1B). The present results show that alteration of one, two, three, or all four Cys residues in SeHAS eliminates the Mg^{2+}-dependent cooperative activation of the enzyme, indicating a requirement for all four Cys residues in the sigmoidal response to Mg^{2+}. However, Cys-null SeHAS shows no sigmoidal behavior, but is still Mg^{2+}-dependent and active. This finding indicates that either the Cys -SH groups are not involved directly in coordination of Mg^{2+} in wildtype SeHAS or that if they do directly coordinate Mg^{2+}, then this function is not required for activity *in vitro*. Although sulfhydryl groups can directly coordinate with some divalent metal ions, a cooperative Mg^{2+}-binding response might also be indirectly mediated if the cluster of Cys residues facilitates a HAS conformation that is able to bind multiple Mg^{2+} ions in a cooperative way.

Although purified SeHAS had little or no activity when Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, or Cu^{2+} were substituted for Mg^{2+}, these same metal ions in the presence of $MgCl_2$ inhibited the synthesis of HA to varying degrees. The Group 2A elements tested (Ca^{2+}, Sr^{2+} and Ba^{2+}) inhibited SeHAS less effectively (K_i values of 2-5 mM) than the transition elements (row 4: Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) tested (K_i values of 0.2-2 mM), indicating that these two cation groups inhibit by different mechanisms. Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} caused the greatest inhibition of SeHAS activity and generally have larger equilibrium binding constants and smaller atomic radii. Although ionic radii are variable properties of ions that can be influenced by factors such as spin, coordination number, and local environment, a general trend is that ionic radii increase within a descending periodic group. Ionic radii (in pm) for the ions used here are: Cu^{2+} (73); Zn^{2+} (75); Co^{2+} (65); Ni^{2+} (70); Mn^{2+} (82); Ba^{2+} (142); Sr^{2+} (125); Ca^{2+} (112). The observed correlation for these ions, which are listed in order of decreasing potency to inhibit SeHAS, is that ionic radius increases as ion inhibition ability decreases. Ionic size, however, does not solely explain overall inhibitory potency, e.g. among the four most potent ions.

Several mechanisms could explain the inhibition of SeHAS activity by divalent metal ions. One mechanism is that some ions displace Mg^{2+} in coordination with the pyrophosphoryl substrates, UDP-GlcUA and UDP-GlcNAc [39] and HA-UDP (in the case of synthesis at the reducing end of HA). A second known mechanism is that some metal ions (e.g. Ag^{+}) can bind covalently to -SH or other groups in proteins [40]. A third established mechanism is the formation of high affinity coordination complexes between a metal ion and unpaired electrons in the orbitals of specific, spatially well positioned atoms in proteins [39,41,42]. Metal ions have known characteristic preferences for binding with particular amino acid R groups and atoms [43]. For example, Mg^{2+}, Mn^{2+}, Ca^{2+}, Cr^{3+} and Co^{3+} are bound mainly to O-containing ligands, whereas Cu^{+}, Ag^{+}, Au^{+}, Ti^{+}, Pd^{2+}, Pt^{2+}, Cd^{2+}, and Hg^{+} are generally bound to S-containing ligands. In contrast, Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, and Co^{2+} preferentially bind to N-containing ligands.

Our results with purified SeHAS and the panel of membrane-bound Cys-mutants lead us to conclude that the first mechanism noted above (displacement of Mg^{2+} in UDP-substrate complexes) accounts for approximately 20-30% inhibition for any SeHAS variant, and is the sole mechanism of action of the least potent Group 2 ions (Ca^{2+}, Sr^{2+} and Ba^{2+}). This is also evident with the Cys-null SeHAS, for which the effect of the most potent inhibitor (Cu^{2+}) on wildtype enzyme was similar to that of Ca^{2+} (Figure 5). The inhibition patterns of the more potent ions, particularly Cu^{2+}, were distinct depending on the combination of the four Cys residues mutated in each SeHAS variant. The results indicate that whether directly or indirectly, all four Cys residues are involved in and required for ligation and high affinity binding of inhibitory ions to the protein. The third mechanism, coordination complex formation, noted above seems the most consistent with our results. One possibility is that the different inhibitory ions may be coordinated not only by one or more -SH groups in the Cys residues but also by other nearby preferred ligation atoms in the active sites. Alternatively, the Cys residues are required for a subtle conformation change that brings a set of coordinating atoms together that allows high affinity binding to appropriate ions (e.g. inhibition of SeHAS by Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+} could be due to the metal ion binding to N-containing amino acids in or near the active site).

Finally, we present a possible explanation for the cooperative response to Mg^{2+} that is speculative, but may be important since it addresses a novel aspect of HA synthase function, namely the lipid dependence of the Class I HAS family [11]. A possible reason for the cooperative activation of enzyme could be that Mg^{2+} ions are required either for the interactions between SeHAS and the multiple CL molecules in an active enzyme-lipid complex or for the ability to tightly pack CL molecules around SeHAS in order to activate it. In *in vitro* liposome systems Mg^{2+} has a strong interaction potential with CL [44] and can induce lamellar-to-inverted phase transitions and membrane fusion [45]. In intact *E. coli* membranes CL microdomains localize to regions of negative curvature [46]. Since CL has two head groups with four fatty acyl chains, its shape is conical and it naturally tends to create negative (concave) membrane curvature. In fact, HAS might be activated by CL because it decreases lateral pressure on the protein [47] and thus opens up the cytoplasmic active sites and intraHAS pore.

The close packing of negatively charged dual CL head groups (that can bind two Mg^{2+} ions), in regions of negative curvature might require a higher Mg^{2+} concentration to saturate formation of Mg^{2+}-CL complexes. Previous radiation inactivation studies revealed that about 16 CL molecules are required for maximal activity of native SpHAS and SeHAS [22]. Thus, fully active HAS species are actually HAS-CL_{16} complexes. Mg^{2+} binding to CL might facilitate formation of successive intermediate HAS●CL_x species. Intermediates approaching a fully activated HAS●CL_{16} complex could become more and more stable, thus lowering the binding energy for the addition of each successive $(Mg^{2+})_2$-CL to the complex and creating a cooperative activation process.

For cells such as *S. equisimilis* or *S. pyogenes* the metabolic burden of continuously making an HA capsule and secreting large amounts of

HA into the medium could be a factor in the evolution of structure-function relationships within their HA synthases. For example, the K_m of HAS for UDP-GlcNAc is relatively high so it does not interfere with the utilization of this import precursor by other glycosytransferases, e.g. for cell wall biosynthesis [37]. Since all cells require substantial Mg^{2+} concentrations for DNA, RNA, phospholipid and nucleotide metabolism, the cooperative activation of streptococcal HASs (thus requiring a higher Mg^{2+} concentration for HAS activity) might ensure that HA synthesis does not occur at the expense of cell survival by depleting too much Mg^{2+}, energy, or sugar units.

This latter effect might have created continued selective pressure to retain the four Cys residues in mammalian HASs as well, since naturally occurring HAS mutants that lose the cooperative activation response to Mg^{2+}, like the mutants used in this study, might impair cell survival or function. Whatever the reason, our results show that the cluster of four highly conserved Cys residues in SeHAS is required for the cooperative response to increasing Mg^{2+} concentration and for the severe inhibition of HAS function by divalent cations such as Cu^{2+}.

Acknowledgements

We thank Jennifer Washburn for technical assistance with these studies. This research was supported by National Institutes of Health grant GM35978 from the National Institute of General Medical Sciences.

References

1. Laurent TC, Fraser JR (1992) Hyaluronan. FASEB J 6: 2397-2404.
2. Knudson CB, Knudson W (1993) Hyaluronan-binding proteins in development, tissue homeostasis, and disease. FASEB J 7: 1233-1241.
3. Markovitz A, Cifonelli JA, Dorfman A (1959) The biosynthesis of hyaluronic acid by group A streptococcus-VI. Biosynthesis from uridine nucleotides in cell-free extracts. J Biol Chem 234: 2343-2350.
4. DeAngelis PL (1999) Hyaluronan synthases: fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses. Cell Mol Life Sci 56: 670-682.
5. Jong A, Wu CH, Chen HM, Luo F, Kwon-Chung KJ, et al. (2007) Identification and characterization of CPS1 as a hyaluronic acid synthase contributing to the pathogenesis of Cryptococcus neoformans infection. Eukaryot Cell 6: 1486-1496.
6. Toole BP (1997) Hyaluronan in morphogenesis. J Intern Med 242: 35-40.
7. Fenderson BA, Stamenkovic I, Aruffo A (1993) Localization of hyaluronan in mouse embryos during implantation, gastrulation and organogenesis. Differentiation 54:85-98.
8. Knudson CB (1993) Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. J Cell Biol 120: 825-834.
9. Nishida Y, Knudson CB, Nietfeld JJ, Margulis A, Knudson W (1999) Antisense inhibition of hyaluronan synthase-2 in human articular chondrocytes inhibits proteoglycan retention and matrix assembly. J Biol Chem 274: 21893-21899.
10. Hall CL, Turley EA (1995) Hyaluronan: RHAMM mediated cell locomotion and signaling in tumorigenesis. J Neurooncol 26: 221-229.
11. Weigel PH, DeAngelis PL (2007) Hyaluronan synthases: A decade-plus of novel glycosyltransferases. J Biol Chem 282: 36777-36781.
12. Heldermon C, DeAngelis PL, Weigel PH (2001) Topological organization of the hyaluronan synthase from Streptococcus pyogenes. J Biol Chem 276: 2037-2046.
13. Weigel PH (2002) Functional characteristics and catalytic mechanisms of the bacterial hyaluronan synthases. IUBMB Life 54: 201-211.
14. Ng KF, Schwartz NB (1989) Solubilization and partial purification of hyaluronate synthetase from oligodendroglioma cells. J Biol Chem 264: 11776-11783.
15. Mian N (1986) Analysis of cell-growth-phase-related vatiations in hyaluronate synthase activity of isolated plasma-membrane fractions of cultured human skin fibroblasts. Biochem J 237: 333-342.
16. Stoolmiller AC, Dorfman A (1969) The biosynthesis of hyaluronic acid by Streptococcus. J Biol Chem 244: 236-246.
17. Triscott MX, van de Rijn I (1986) Solubilization of hyaluronic acid synthetic activity from streptococci and its activation with phospholipids. J Biol Chem 261: 6004-6009.
18. Tlapak-Simmons VL, Baggenstoss BA, Clyne T, Weigel PH (1999) Purification and lipid dependence of the recombinant hyaluronan synthases from Streptococcus pyogenes and Streptococcus equisimilis. J Biol Chem 274: 4239-4245.
19. Yoshida M, Itano N, Yamada Y, Kimata K (2000) In vitro synthesis of hyaluronan by a single protein derived from mouse HAS1 gene and characterization of amino acid residues essential for the activity. J Biol Chem 275: 497-506.
20. Tlapak-Simmons VL, Baggenstoss BA, Kumari K, Heldermon C, Weigel PH (1999) Kinetic characterization of the recombinant hyaluronan synthases from Streptococcus pyogenes and Streptococcus equisimilis. J Biol Chem 274: 4246-4253.
21. Tlapak-Simmons VL, Baron CA, Weigel PH (2004) Characterization of the purified hyaluronan synthase from Streptococcus equisimilis. Biochem 43: 9234-9242.
22. Tlapak-Simmons VL, Kempner ES, Baggenstoss BA, Weigel PH (1998) The active streptococcal hyaluronan synthases (HASs) contain a single HAS monomer and multiple cardiolipin molecules. J Biol Chem 273: 26100-26109.
23. Kumari K, Weigel PH (2005) Identification of a membrane-localized cysteine cluster near the substrate binding sites of the streptococcus equisimilis hyaluronan synthase. Glycobiology 15: 529-539.
24. Prehm P (1983) Synthesis of hyaluronate in differentiated teratocarcinoma cells. Mechanism of chain growth. Biochem J 211: 191-198.
25. Tlapak-Simmons VL, Baron CA, Gotschall R, Haque D, Canfield WM, et al. (2005) Hyaluronan biosynthesis by Class I streptococcal hyaluronan synthases occurs at the reducing end. J Biol Chem 280: 13012-13018.
26. Bodevin-Authelet S, Kusche-Gullberg M, Pummill PE, DeAngelis PL, Lindahl U (2005) Biosynthesis of hyaluronan: Direction of chain elongation. J Biol Chem 280: 8813-8818.
27. Kumari K, Weigel PH (1997) Molecular cloning, expression, and characterization of the authentic hyaluronan synthase from group C Streptococcus equisimilis. J Biol Chem 272: 32539-32546.
28. Kumari K, Tlapak-Simmons VL, Baggenstoss BA, Weigel PH (2002) The streptococcal hyaluronan synthases are inhibited by sulfhydryl modifying reagents but conserved cysteine residues are not essential for enzyme function. J Biol Chem 277: 13943-13951.
29. Heldermon CD, Tlapak-Simmons VL, Baggenstoss BA, Weigel PH (2001) Site-directed mutation of conserved cysteine residues does not inactivate the Streptococcus pyogenes hyaluronan synthase. Glycobiology 11: 1017-1024.
30. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
31. DeAngelis PL, Papaconstantinou J, Weigel PH (1993) Molecular cloning, identification and sequence of the hyaluronan synthase gene from group A streptococcus pyogenes. J Biol Chem 268: 19181-19184.
32. Hill AV (1913) The combinations of haemoglobin with oxygen and with carbon monoxide. Biochem J 7: 471-480.
33. Pummill PE, Achyuthan AM, DeAngelis PL (1998) Enzymological characterization of recombinant Xenopus DG42, a vertebrate hyaluronan synthase. J Biol Chem 273: 4976-4981.
34. Itano N, Kimata K (2002) Mammalian hyaluronan synthases. IUBMB Life 54: 195-199.
35. Baggenstoss BA, Weigel PH (2006) Size exclusion chromatography-multiangle laser light scattering analysis of hyaluronan size distributions made by membrane-bound hyaluronan synthase. Anal Biochem 352: 243-251.
36. DeAngelis PL, Weigel PH (1994) Immunochemical confirmation of the primary structure of streptococcal hyaluronan synthase and synthesis of high molecular weight product by the recombinant enzyme. Biochem 33: 9033-9039.
37. Weigel PH (1998) Bacterial hyaluronan synthases. Hascall, V. C. and Yanagishita, M. Science of Hyaluronan Today. JGL format for website article?
38. Unligil UM, Rini JM (2000) Glycosyltransferase structure and mechanism. Curr Opin Struct Biol 10: 510-517.

39. Lairson LL, Henrissat B, Davies GJ, Withers SG (2008) Glycosyltransferases: structures, functions, and mechanisms. Annu Rev Biochem 77: 521-555.

40. Giles NM, Watts AB, Giles GI, Fry FH, Littlechild JA, et al. (2003) Metal and redox modulation of cysteine protein function. Chem Biol 10: 677-693.

41. Picard M, Jensen AM, Sorensen TL, Champeil P, Moller JV, et al. (2007) Ca2+ versus Mg2+ coordination at the nucleotide-binding site of the sarcoplasmic reticulum Ca2+-ATPase. J Mol Biol 368: 1-7.

42. Quiocho FA (1986) Carbohydrate-binding proteins: tertiary structures and protein-sugar interactions. Annu Rev Biochem 55: 287-315.

43. Glusker JP (1991) Structural aspects of metal liganding to functional groups in proteins. Adv Protein Chem 42: 1-76.

44. Merchant TE, Glonek T (1992) 31P NMR of tissue phospholipids: competition for Mg2+, Ca2+, Na+ and K+ cations. Lipids 27: 551-559.

45. Ortiz A, Killian JA, Verkleij AJ, Wilschut J (1999) Membrane fusion and the lamellar-to-inverted-hexagonal phase transition in cardiolipin vesicle systems induced by divalent cations. Biophys J 77: 2003-2014.

46. Renner LD, Weibel DB (2011) Cardiolipin microdomains localize to negatively curved regions of Escherichia coli membranes. Proc Natl Acad Sci U S A 108: 6264-6269.

47. Huang, KC, Ramamurthi KS (2010) Macromolecules that prefer their membranes curvy. Mol Microbiol 76: 822-832.