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Individual Smad2 linker region phosphorylation sites determine the expression of proteoglycan and glycosaminoglycan synthesizing genes

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ABSTRACT

Growth factors such as thrombin and transforming growth factor (TGF)-β facilitate glycosaminoglycan (GAG) chain hyperelongation on proteoglycans, a phenomenon that increases lipoprotein binding in the vessel wall and the development of atherosclerosis. TGF-β signals via canonical carboxy terminal phosphorylation of R-Smads and also non-canonical linker region phosphorylation of R-Smads. The G protein coupled receptor agonist, thrombin, can transactivate the TGF-β receptor leading to both canonical and non-canonical Smad signalling. Linker region phosphorylation drives the expression of genes for the synthesis of the proteoglycan, biglycan. Proteoglycan synthesis involves core protein synthesis, the initiation of GAG chains and the subsequent elongation of GAG chains. We have explored the relationship between the thrombin stimulated phosphorylation of individual serine and threonine sites in the linker region of Smad2 and the expression of GAG initiation xylosyltransferase-1 (XT-1) and GAG elongation chondroitin 4-sulfotransferase-1 (C4ST-1) and chondroitin synthase-1 (CHSY-1) genes. Thrombin stimulated the phosphorylation of all four target residues (Thr220, Ser245, Ser250 and Ser255 residues) with a similar temporal pattern – phosphorylation was maximal at 15 min (the earliest time point studied) and the level of the phosphoproteins declined thereafter over the following 4 h. Jnk, p38 and PI3K, selectively mediated the phosphorylation of the Thr220 residue whereas the serine residues were variously phosphorylated by multiple kinases. Thrombin stimulated the expression of all three genes – XT-1, C4ST-1 and CHSY-1. The three pathways mediating Thr220 phosphorylation were also involved in the expression of XT-1. The target pathways (excluding Jnk) were involved in the expression of the GAG elongation genes (C4ST-1 and CHSY-1). These findings support the contention that individual Smad linker region phosphorylation sites are linked to the expression of genes for the initiation and elongation of GAG chains on proteoglycans. The context of this work is that a specific inhibitor of GAG elongation represents a potential therapeutic agent for preventing GAG elongation and lipid binding and the results indicate that the specificity of the pathways is such that it might be therapeutically feasible to specifically target GAG elongation without interfering with other physiological processes with which proteoglycans are involved.

1. Introduction

Transforming growth factor (TGF)-β is a pleiotropic growth factor with broad involvement in physiological and pathophysiological processes. TGF-β has a very strong effect to stimulate the hyperelongation of glycosaminoglycan (GAG) chains on the major lipid binding

Abbreviations: CDK, Cyclin dependent kinase; CHSY-1, Chondroitin synthase; C4ST-1, Chondroitin 4-O-sulfotranferase; EGFR, Epidermal growth factor receptor; GAG, Glycosaminoglycan; GalT-1, β-1,3-galactosyl transferase-1.; GlcAT-1, β-1,3-glucuronosyl transferase-1; GPCRs, G protein coupled receptors; MAPK, Mitogen activated protein kinase; PI3K, Phosphatidylinositide 3-kinase; PAR, Protease activated receptor; PTKR, Protein tyrosine kinase receptors; S/TKR, Serine/threonine kinase receptors; TGF-β, Transforming growth factor beta; TGFBR1, Transforming growth factor beta receptor; VSMCs, Vascular smooth muscle cells; XT-1, Xylosyltransferase 1

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proteoglycan biglycan and as it is associated with lipid deposition in atherosclerosis then this process represents a potential therapeutic target [1,2]. TGF-β signals via canonical carboxy terminal phosphorylation of R-Smads (Smad2 in vascular smooth muscle cells (VSMC)) and non-canonical linker region phosphorylation of R-Smads [3,4]. The role of TGF-β signalling in cell biology was greatly expanded when we showed that the paradigm of G Protein Coupled Receptor (GPCR) transactivation of protein tyrosine kinase receptors extended to the transactivation of serine/threonine kinase receptors and specifically the Type I TGF-β Receptor (TGFBR1) [5–8].

GPCRs such as protease activated receptor (PAR)-1 and endothelin receptors can engage TGFBR1 and activate downstream signalling commencing with Smad phosphorylation [9–12]. The PAR-1 agonist thrombin can transactivate the TGFBR1 in VSMCs leading to both canonical and non-canonical Smad signalling and downstream effects on the synthesis, specifically elongation of the GAG chains on the lipid-binding chondroitin sulfate/dermatan sulfate (CS/DS) proteoglycan, biglycan. Proteoglycan synthesis is a complex process which involves core protein synthesis, the initiation of GAG chain synthesis and the subsequent elongation and maturity of GAG chains [13–17]. Whereas Smad linker region phosphorylation was initially described to inhibit Smad nuclear translocation [18] and hence TGF-β signalling, in an in vitro model of atherosclerosis we showed that linker region phosphorylation drives the expression of genes for the enzymes and transferases responsible for the synthesis of the biglycan [19–21].

Chondroitin sulfate GAG chain synthesis commences with the synthesis of the tetra-saccharide linkage region by the initial action of xylosyltransferase 1 (XT-1) [22] to transfer a xylose to the serine residue on the core protein, followed by the combined action of three enzymes β-1,3-galactosyl transferase-1 (GalT-1), GalT-2 and β-1,3-glucuronosyl transferase-1 (GlcAT-1) to add two galactose and one glucuronic acid residues. GAG chain synthesis and elongation involves the combination of more than one GAG synthesizing enzyme. An increase in GAG chain length and an increase in atherosclerotic lesion progression in vivo is associated with an increase in GAG enzyme expression [23].

The cell signalling mechanisms involved in GAG synthesis and elongation are distinct [6,24]. The canonical TGF-β signalling pathway comprises direct carboxy-terminal phosphorylation of Smad2 by TGFBR1. In addition, Smads can also be phosphorylated in the linker region [3,4,25,26]. Smad linker region is phosphorylated via multiple serine/threonine kinases including mitogen activated protein kinase (MAPK) (Erk, Jnk and p38), phosphatidylinositide 3-kinase (PI3K) and cyclin dependent kinase (CDK) [19,20,27]. There are multiple phosphorylation sites in the human Smad2 linker region: threonine at position 220 (Thr220) and three serine residues at positions 245, 250, 255 (Ser245, Ser250, Ser255) [3,26]. TGF-β mediated GAG chain hyperelongation [20] and GAG synthesizing gene expression [19] are regulated by the Smad2 linker region. The role of Smad2 linker region phosphorylation in thrombin signalling has not been elucidated but it represents an interesting model to study the implications of TGFBR1 transactivation and the broader relationship between GPCRs and cell surface kinase receptor transactivation.

The Smad2 linker region plays a central integrating point for GPCR mediated signalling for the expression of the genes for the enzymes, which mediate the initiation, and elongation of GAGs on proteoglycans. With the complexity of the synthesis of proteoglycans involving more than ten transferases and elongation factors, this is a suitable model to study the relationship between the individual sites of phosphorylation of serine/threonine residues in the linker region of Smad2 and the resultant expression of specific genes for GAG initiation and elongation transferases.

2. Materials and methods

2.1. Materials

Foetal bovine serum (FBS) was purchased from Invitro Technologies Pty. Ltd. (VIC, Australia). Dulbecco's Modified Eagle Medium (DMEM) (0 mM and 25 mM glucose), Trypsin-Versene, penicillin and streptomycin were from GIBCO BRL (Ne, USA). Human TGF-β, anti-rabbit IgG horseradish peroxidase (HRP) and anti-GAPDH were purchased from Cell Signalling Technology (MA, USA). Flavopiridol, SP600125, SB202190 and LY294002 were purchased from Sigma-Aldrich (St Louis, USA). UO126 was from Promega (WI, USA). CHSY-1 rabbit polyclonal antibody was purchased from Abcam (Vic, Australia). C4ST-1 rabbit polyclonal antibody was purchased from ThermoFishes Scienctific (Vic, Australia). Amersham ECL Prime chemiluminescence detection reagent was purchased from GE Healthcare (Paramatta, Australia). The primers (forward and reverse) for C4ST-1, CHSY-1, XT-1, EXT-1, EXT-2, EXT-3, NDST-1 and 18S, RNeasy Mini Kit, the QuantiTect reverse transcription kit, QuantiFast SYBR green PCR kit and the Rotor Gene Q series software were from Qiagen (Vic, Australia). Anti-phospho-Smad2L (Thr220) rabbit IgG polyclonal, anti-phospho-Smad2L(Ser245) rabbit IgG polyclonal, anti-phospho-Smad2L(Ser250) rabbit IgG polyclonal and anit-phospho-Smad2L(Ser255) rabbit IgG polyclonal were a gift from Professor Koichi Matsuzaki (Kansai Medical University, Osaka, Japan).

2.2. Cell culture

Primary cultures of human VSMCs were obtained from discarded sections of saphenous veins from patients undergoing surgery at the Alfred Hospital (Melbourne, Australia) the acquisitions of the vessels was approved by the Alfred Hospital Ethics Committee. VSMCs were grown in DMEM (5 mM glucose, 10% FBS and 1% antibiotics at 37 °C in 5% CO_2). VSMCs were seeded in 60 mm dishes and 6 well-plates. Cells were grown to confluence then rendered quiescent by serum deprivation for 48 h. Inhibitors were pre-incubated for 30 min prior to treatment with agonists. Incubation times and concentrations are given in detail in the figure legends.

2.3. Western blotting

Whole cell lysates (30 μg of protein) were resolved on 10% SDS-PAGE and semi-dry transferred onto PVDF. Membranes were blocked with 5% bovine serum albumin and incubated with primary antibody targeting protein of interest followed by HRP-anti-rabbit IgG and ECL detection. The protein of interest was normalised with GAPDH to determine equal loading. Blots were imaged using the Bio-Rad gel documentation system and densitometry analysis was performed with Quantity One imaging software.

2.4. Assessing mRNA gene expression

The mRNA level of the GAG enzymes was determined by quantitative real-time polymerase chain reaction (RT-PCR). Total RNA was isolated from VSMCs treated as described. RNA was extracted from 5×10^5 cells using RNeasy Mini kit (Qiagen) according to the manufacturers' instructions. RNA purity was checked by spectrophotometry (260/280 nm) using Nanodrop2000 spectrophotometer (Thermo Fisher Scientific). First strand cDNA was synthesized from 1 μg RNA using Quantitect reverse transcriptase kit (Qiagen) according to the manufacturers' instructions. Quantitative RT-PCR was performed using Qiagen Rotor Gene Q and QuantiFast SYBR green PCR master mix kit (Qiagen). Data was normalised to the ribosomal 18S housekeeping gene to adjust for control variations between individual experiments. Relative expression of mRNA levels was quantified using comparative ΔΔ Ct method. Experiments were performed in duplicate at least three

times.

2.5. Statistical analysis

Normalised data is expressed as the mean ± standard error of the mean of three independent experiments, unless stated otherwise. A one-way ANOVA was used to calculate statistical significance of normalised data as stated followed by least significant difference *post-hoc* analysis. Results were considered significant when the probability was < 0.05 (*$p < .05$) and 0.01 (**$p < .01$).

3. Results

3.1. Thrombin stimulates the phosphorylation of individual Smad2 linker region residues in VSMCs

There are at least four important phosphorylation sites on the human Smad2 linker region being Thr220, Ser245, Ser250 and Ser255 [3]. To determine whether site-specific linker region phosphorylation is mediated by thrombin signalling in VSMCs, a time course study was preformed with antibodies specific for the individual phosphorylated linker region residues [28]. Thrombin treated VSMCs showed a rapid increase in the phosphorylation of the Thr220 residue. Thrombin mediated phosphorylation peaked at 15 min with a 2.5-fold ($p < .01$) change when compared to basal (Fig. 1A). Phosphorylation was sustained although there was a gradual decline over the 4 h treatment. There was also a rapid increase in the phosphorylation of Ser245 reaching a peak of 1.9-fold ($p < .01$) at 15 min when compared to basal (Fig. 1B). This was sustained for 4 h. Similarly the Ser250 residue was rapidly phosphorylated in the presence of thrombin with a peak phosphorylation of 2.5-fold ($p < .01$) at 15 min (Fig. 1C). The phosphorylation of this residue was sustained for 60 min. The phosphorylation of the Ser255 residue followed the same trend as the other linker region residues in which thrombin caused a rapid increase with a peak at 15 min of 2.5-fold ($p < .01$) when compared to basal and this gradually declined over 4 h. These results demonstrate that in VSMCs thrombin treatment rapidly phosphorylates Thr220, Ser245, Ser250 and Ser255 in the Smad2 linker region. The challenge then is to determine the kinases mediating these specific phosphorylations.

3.2. The involvement of serine/threonine kinases in thrombin stimulated phosphorylation of Smad2 linker region residues

Smad2 linker region phosphorylation involves activation of serine/threonine kinases. Here we investigated kinases Erk, p38, Jnk, PI3K and CDK on thrombin phosphorylation of individual Smad2 linker region residues. VSMCs treated with thrombin for 30 min increased the phosphorylation of the Thr220 to 2.7-fold ($p < .01$) (Fig. 2A) which was unaffected in the presence of Erk and CDK inhibitors, UO126 and flavopiridol. Inhibitors to p38 and Jnk, SB202190 and SP600124 partially inhibited thrombin mediated phosphorylation of Thr220 and in the presence of PI3K inhibitor, LY294002 this response was completely inhibited (Fig. 2A). These results demonstrate that phosphorylation of the Thr220 residue via thrombin dependent mechanisms involves p38, Jnk and PI3K pathways but not Erk and CDK.

Thrombin treatment of VSMCs caused an increase in the phosphorylation of Ser245 to 2-fold ($p < .01$) at 30 min (Fig. 2B). The presence of inhibitors to Erk, p38 and Jnk had no effect on the thrombin mediated phosphorylation of Ser245. PI3K inhibitor, partially inhibited thrombin mediated phosphorylation of Ser245, and in the presence of

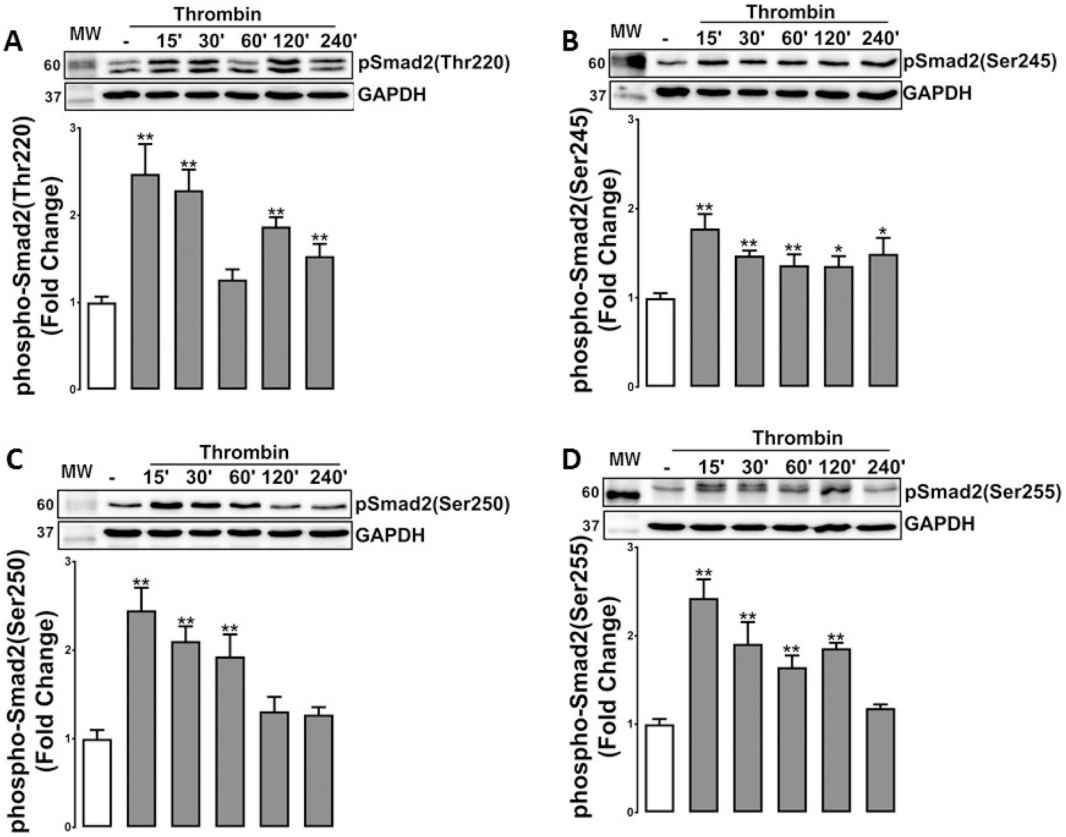

Fig. 1. Time course of thrombin mediated phosphorylation of four specific Smad2 linker region residues. Western blots probed with antibodies specific to **A** phospho-Smad2/3(Thr220/179) **B** phospho-Smad2(Ser245) **C** anti-phospho-Smad2(Ser250) and **D** anti-phospho-Smad2(Ser255). Blots shown are representative of three independent experiments. Histograms represent band density expressed as fold per basal from three independent experiments (*p < .05 vs basal) (**p < .01 vs basal) using one-way ANOVA followed by least significant difference *post-hoc* analysis.

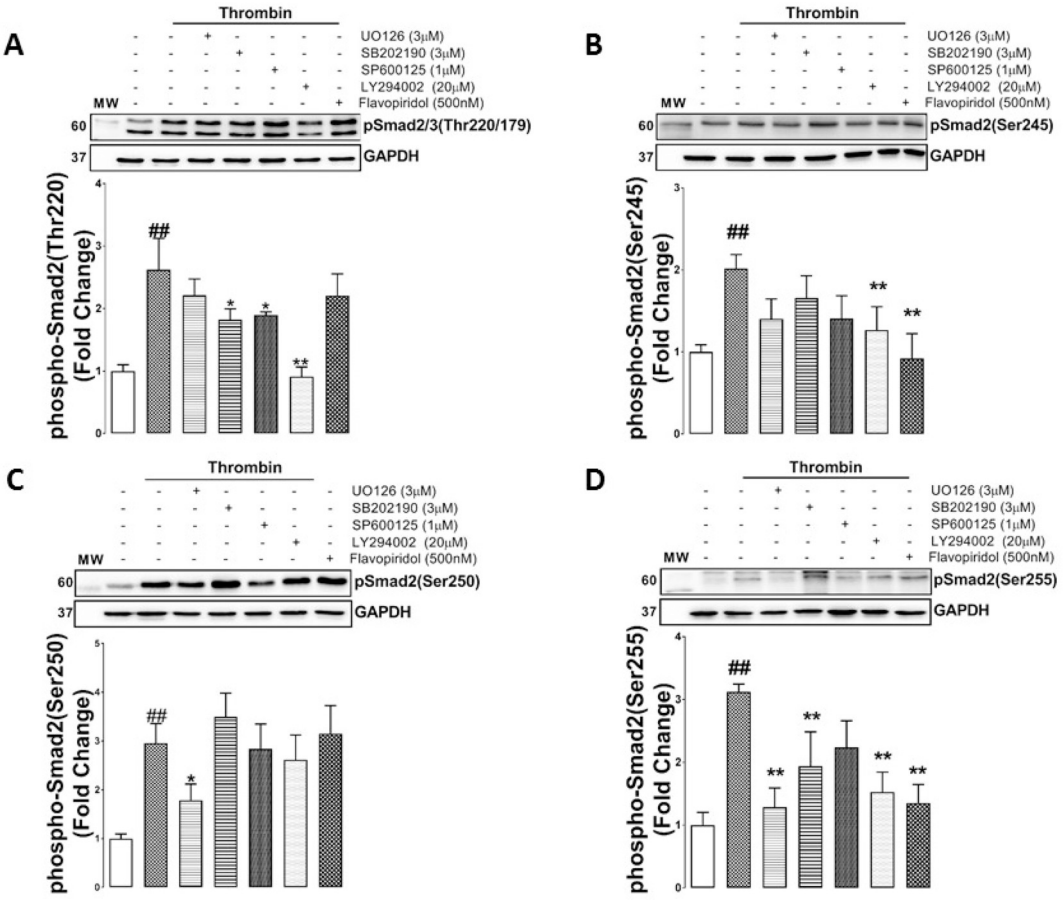

Fig. 2. Effects of inhibitors to Erk, p38, Jnk, CDK and PI3K on thrombin mediated Smad2 linker phosphorylation. VSMCs were treated with thrombin for 15 min in the presence and absence of each of the serine/threonine kinase inhibitors. Each blot was probed with antibodies specific to **A.** phospho-Smad2/3(Thr220/179) **B.** phospho-Smad2(Ser245) **C.** phospho-Smad2(Ser250) and **D.** phospho-Smad2(Ser255). Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis ##, $p < .01$ versus basal and **, $p < .01$ agonist versus antagonist.

CDK inhibitor there was complete inhibition ($p < .01$) (Fig. 2B). Thus, thrombin stimulated Ser245 phosphorylation requires the activation of PI3K and CDK.

In cells treated with thrombin, the level of the phosphorylation of Smad2 linker region residue Ser250 was increased to almost 3-fold ($p < .01$) (Figs. 2C). Erk inhibitor, UO126 partialy inhibited Ser250 phosphorylation to 2-fold ($p < .05$). In the presence of inhibitors to p38, Jnk, PI3K and CDK, thrombin mediated phosphorylation of Ser250 is unnaffected (Fig. 2C). Thus showing that Erk is the only kinase involved in thrombin mediated phosphorylation of Smad2 linker region residue Ser250.

Thrombin mediated phosphoryaltion of the Ser255 residue was completely inhibited in the presence of UO126 ($p < .01$) (Fig. 2D). SB202190, LY294002 and flavopiridol partially inhibited this response however SP600125 had no effect (Fig. 2D). Thus showing that thrombin mediated phosphorylation of Ser255 occurs via the activation of the Erk, p38, PI3K and CDK pathways. Taken together the results demonstrate that individual Smad2 linker region residues are phosphorylated by different serine/threonine kinases, with the PI3K being a common kinase for Thr220, Ser245 and Ser255 and Jnk is involved in regulating the Thr220 residue.

3.3. Thrombin stimulates the expression of genes associated with GAG initiation (XT-1) and elongation of CS GAG chains (C4ST-1 and CHSY-1)

To investigate if thrombin can stimulate the mRNA expression of GAG biosynthesizing genes, specifically CHSY-1, C4ST-1 and XT-1, VSMCs were treated with thrombin (10 units/ml) and the mRNA expression of these genes was measured over a 24 h time period (Fig. 3). During the biosynthesis of GAG chains, GalNAc residues of the repeating disaccharide units are sulfated at C4 by C4ST-1. Treatment of VSMCs with thrombin generated a parabolic time dependent increase in the mRNA expression of C4ST-1 (Fig. 3A). At 2 h post thrombin treatment, a 1.4-fold increase ($p < .05$) in the mRNA expression of C4ST-1 was detected and reached a peak of 3-fold at 6–8 h post stimulation ($p < .01$) (Fig. 3A). The addition of a monosaccharide to an acceptor oligosaccharide is referred to as chondroitin polymerization which occurs with the action of glycosyltransferase CHSY-1 [14]. Thrombin mediated mRNA expression of CHSY-1 followed a similar pattern to the expression of C4ST-1 where it caused a parabolic time dependent increase in the mRNA expression (Fig. 3B). Thrombin treated VSMCs caused a 2-fold increase ($p < .05$) in the mRNA expression of CHSY-1 at 4 h and this was sustained reaching a peak of 3-fold ($p < .01$) at 8 h (Fig. 3B).

XT-1 is an enzyme that catalyses the attachment of xylose, the first residue of the linkage region that links the CS GAG chain backbone to proteoglycan core protein. When examining the mRNA expression of XT-1 we observed a slight increase of 1.8-fold ($p < .01$) at 4 h post treatment (Fig. 3C). However from 4 h onwards there was no effect on the mRNA expression of XT-1. Together these results demonstrate that thrombin treatment of VSMCs leads to an increase in the mRNA expression of GAG chain initiation gene XT-1 and GAG elongation genes C4ST-1 and CHSY-1.

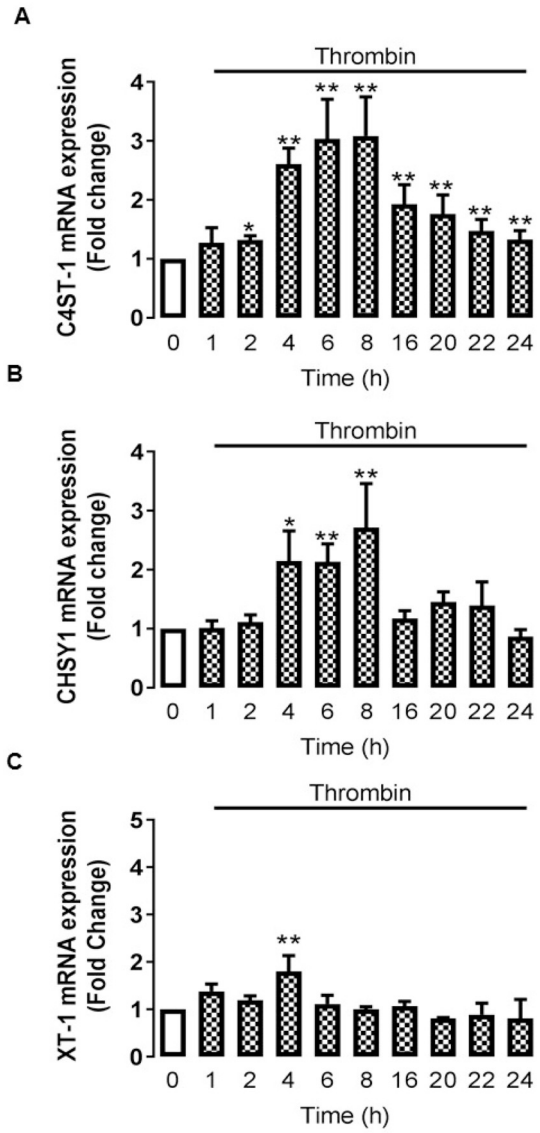

Fig. 3. Time course of thrombin mediated mRNA expression of GAG synthesizing genes C4ST-1, CHSY-1 and XT-1. VSMCs were treated with thrombin (10 units/ml) to investigate the effect of the mRNA expression of **A** C4ST-1, **B** CHSY-1 and **C** XT-1. Total RNA was harvested, cDNA was synthesized and the mRNA expression of these genes was analysed using quantitative RT-PCR. 18S was used as a house keeping gene. Results are expressed as the mean ± standard error from three independent experiments. Statistical significance was determined by one-way ANOVA, followed by the least significance post hoc analysis, $^{**}p < .01$ and $^{*}p < .05$ basal versus agonist.

3.4. Thrombin stimulates the synthesis of C4ST-1 and CHSY1 proteins

The protein expression of C4ST-1 and CHSY-1 was measured using western blotting to investigate whether the increase in the mRNA expression resulted in the increase in the protein expression of the respective GAG synthesizing enzyme. Treatment with thrombin stimulated the synthesis of C4ST-1 from 16 to 24 h (Fig. 4A). The peak protein expression of C4ST-1 of 2-fold ($p < .01$) was observed at 20 h. Thrombin treatment stimulated the expression of CHSY-1. An increase protein expression of CHSY-1 was observed from 16 to 24 h ($p < .01$) (Fig. 4B) with a peak of 2-fold reached at 20 h. Taken together these results demonstrate that the change in mRNA expression of C4ST-1 and CHSY-1 correlated with the protein expression of the respective enzymes.

3.5. Thrombin regulates the mRNA expression of genes associated with the elongation of heparin sulphate GAG chains

GAG chain synthesis requires the combined action of enzymes associated with polymerization and sulphation [29]. To investigate whether thrombin is involved in regulating the genes associated with the elongation of heparin sulphate GAG chains the mRNA expression of extostatin-like glycosoltransferase (EXT)-1,2,3 and N-sulfotransferase-1 (NDST-1) was measured over an 8 h time period (Fig. 5). Treatment of VSMCs with thrombin generated a modest increase in the mRNA expression of EXT1 to 1.5 fold ($p < .05$)(Fig. 5A) at 6 h. VSMCs treated with thrombin had no effect on the mRNA expression of EXT2 (Fig. 5B). However thrombin treatment generated an increase in the mRNA expression of the EXT3 enzyme (Fig. 5C). A gradual stimulation was observed at 2 h, which reached a peak stimulation of 1.4-fold ($p < .01$) (Fig. 5C) at 6–8 h with thrombin treatment. NDST-1 involved in the sulphation of the heparin GAG chain was investigated. Treatment with thrombin over an 8 h period had no effect on the mRNA levels of NDST-1. Taken toghether these results show that thrombin plays a modest role in regulating heparin sulphate synthezing enzymes EXT1 and EXT2 and is not involved in regulating EXT2 and NDST-1 expression in VSMCs.

3.6. Serine/threonine kinases differentially regulate thrombin mediated mRNA expression of genes involved in the initiation and elongation of GAG chains

Our final aim was to evaluate the downstream consequences of Smad2 linker phosphorylation by measuring the expression of genes involved in GAG synthesis. Smad2 linker region phosphorylation occurs via cytosolic serine/threonine kinases [25]. Hence, we hypothesized that the serine/threonine kinases involved in phosphorylation of the Smad2 linker region will regulate thrombin mediated mRNA expression of C4ST-1 and CHSY-1.

VSMCs treated with thrombin for 6 h showed a 2.9-fold increase in the mRNA expression of C4ST-1 compared to non-treated cells (Fig. 6A). In the presence of UO126, SB202190, LY294002 and flavopiridol thrombin stimulated mRNA expression of C4ST-1 was almost completely inhibited (Fig. 6A). In the presence of the Jnk inhibitor SP600125 there was no inhibition of C4ST-1 mRNA expression. This data shows that Erk, p38, PI3K and CDK pathways are involved in thrombin stimulation of C4ST-1 mRNA expression.

Thrombin treatment of VSMCs showed a 2-fold increase of CHSY-1 mRNA expression (Fig. 6B). Thrombin mediated mRNA expression of CHSY-1 was inhibited in the presence of Erk, p38, PI3K and CDK inhibitors (Fig. 6B). Thrombin stimulated CHSY-1 mRNA expression was not blocked by Jnk inhibitor, SP600125. These data demonstrate that thrombin stimulation of CHSY-1 mRNA expression involved Erk, p38, PI3K and CDK but not Jnk. The serine/threonine kinases involved in thrombin mediated mRNA expression of GAG enzymes C4ST-1 and CHSY-1 are the same for both genes.

Thrombin mediated mRNA expression of C4ST-1 and CHSY-1 was not regulated by Jnk. We have previously demonstrated that Jnk, was not involved in TGF-β mediated proteoglycan synthesis and GAG elongation however its role in GAG chain initiation has not yet been investigated [20]. Our earlier results (Fig. 2) showed that the Jnk inhibitor SP600125 has no effect on thrombin mediated phosphorylation of the serine residues (Ser245/250/255). However Jnk played a role in thrombin mediated phosphorylation of the Thr220 residue. Here we explored the role of the kinases which regulate the Thr220 residue and their role in the initiation of the GAG chain by studying mRNA expression of XT-1. VSMCs treated with thrombin showed a 3 fold increase in the mRNA expression of XT-1 (Fig. 6C). In the presence of SP600125, LY294002 and SB202190 thrombin mediated mRNA expression of XT-1 was inhibited by approximately 50% to 2 fold (Fig. 6C). This data shows that the Jnk, PI3K and p38 are involved in thrombin mediated mRNA expression of XT-1 and GAG chain initiation

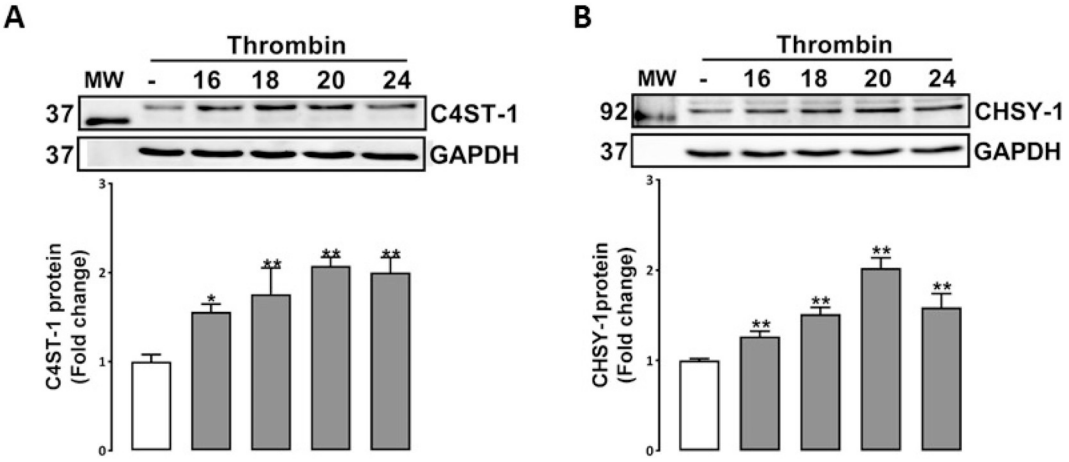

Fig. 4. Thrombin time course of GAG synthesizing enzyme C4ST-1 and CHSY-1 protein expression. VSMCs were treated with thrombin (10 units/ml) for 16, 18, 20 and 24 h. Western blots probed with antibodies specific to **A** C4ST-1 and **B** CHSY-1. Blots shown are representative of three independent experiments. Histograms represent band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA followed by least significant difference *post-hoc* analysis ** $p < .01$ versus non treated basal.

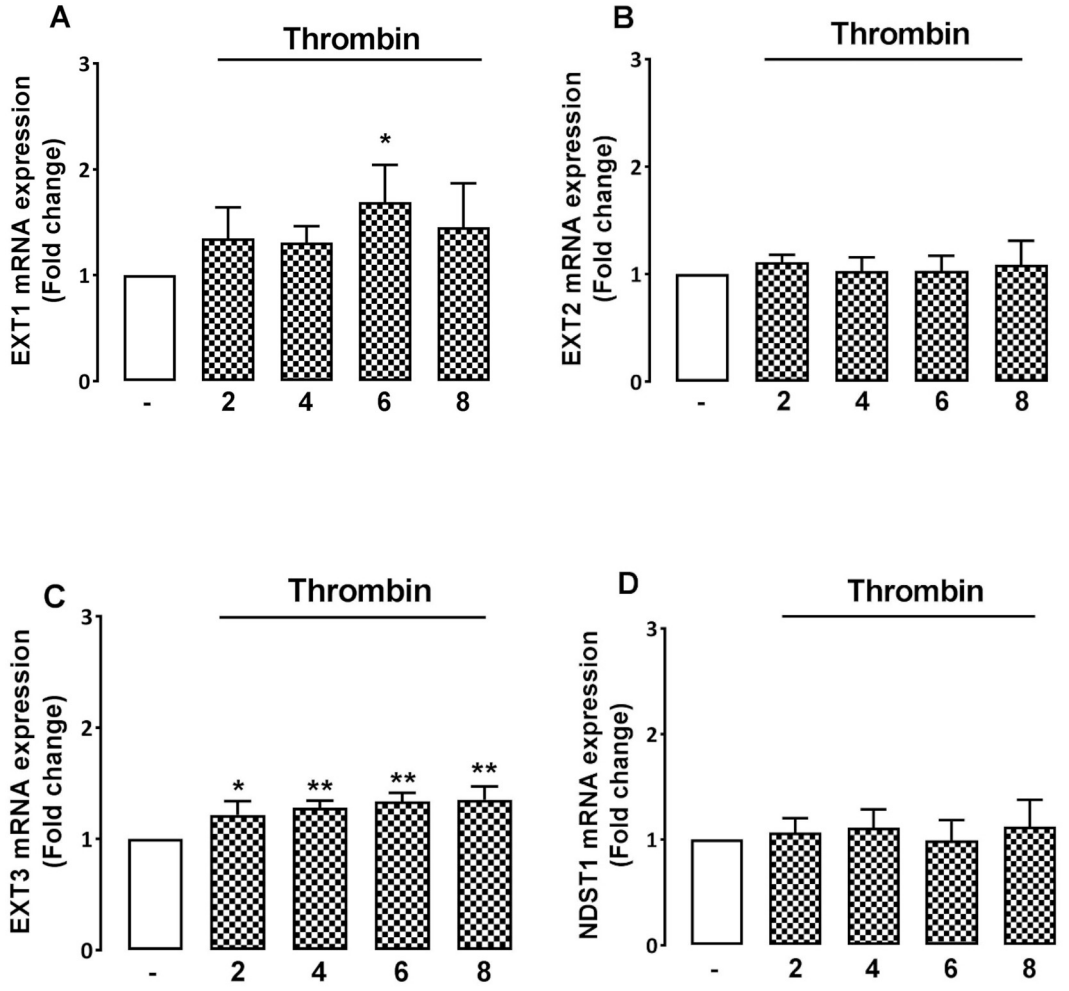

Fig. 5. Time course of thrombin mediated mRNA expression of heparin sulfate synthesizing genes EXT1, EXT2, EXT3 and NDST1. VSMCs were treated with thrombin (10 units/ml) to investigate the effect of the mRNA expression of **A** EXT1, **B** EXT2, **C** EXT3 and **D** NDST1. Total RNA was harvested, cDNA was synthesized and the mRNA expression of these genes was analysed using quantitative RT-PCR. 18S was used as a house keeping gene. Results are expressed as the mean ± standard error from three independent experiments. Statistical significance was determined by one-way ANOVA, followed by the least significance post hoc analysis, **p < .01 and * p < .05 basal versus agonist.

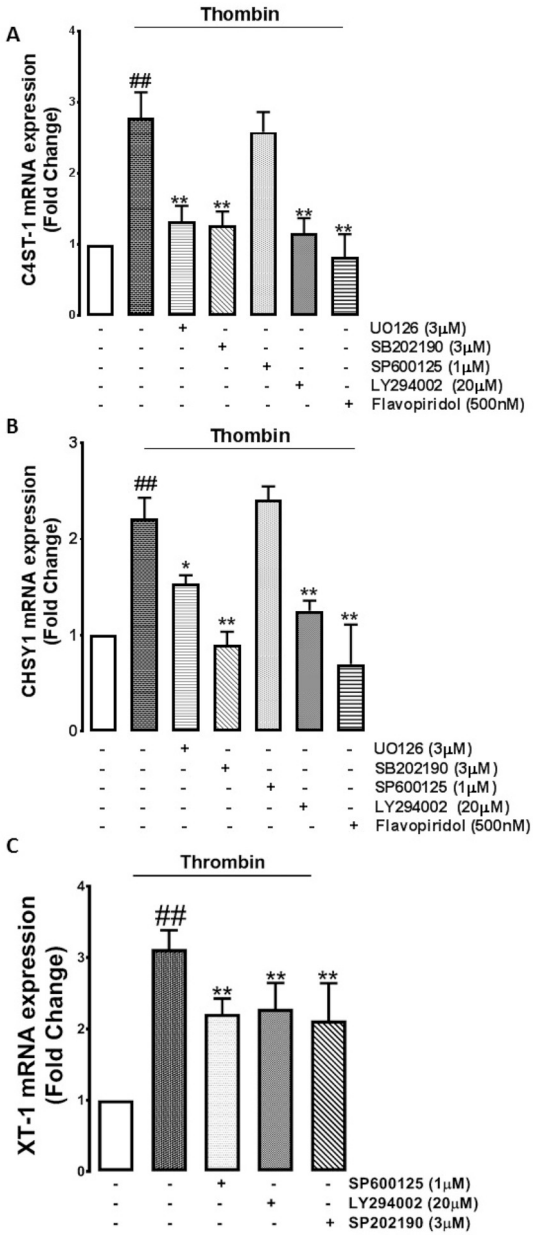

Fig. 6. Serine/threonine kinases differentially regulate thrombin mediated mRNA expression of GAG synthesizing enzymes. VSMCs were pretreated with serine/threonine kinase inhibitors in the presence of thrombin to determine the effects on the mRNA expression of **A.** C4ST-1, **B.** CHSY-1 and **C.** XT-1. Data are expressed as the mean ± standard error from three experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. ##p < .01 basal versus thrombin and *p < .01 and **p < .01 thrombin versus antagonist was used for statistical analysis.

demonstrating the exquisite specificity of the signalling pathways regulating the synthesis of a single molecule – biglycan.

4. Discussion

Proteoglycan core protein synthesis as well as the hyperelongation of GAG chain results in increased binding to LDL causing increased lipid retention and accelerated atherosclerosis [2,30]. Signalling pathways regulating proteoglycan synthesis and GAG chain elongation have therapeutic potential as targets for early onset of atherosclerosis [31]. Thrombin signalling pathways leads to proteoglycan synthesis, GAG elongation [10] and an increase in the mRNA expression of GAG synthesizing genes [11]. TGF-β mediated proteoglycan synthesis [20,32] and GAG gene expression [19,21] are regulated by the phosphorylation of transcription factor Smad2 in the linker region. Multiple signalling pathways are involved in Smad2 linker region phosphorylation [3,8]. In this study we investigated the role of the phosphorylation of individual Smad2 linker region residues in thrombin mediated expression of GAG synthesizing genes involved in the initiation and elongation of GAG chains.

The key findings of this study are that in VSMCs, treatment with thrombin can rapidly increase the phosphorylation of the 4 residues of the Smad2 transcription factors in its linker region. Thrombin treatment activated Erk, p38, PI3K and CDKs but not Jnk to mediate GAG chain hyperelongation measured as CHSY-1 and C4ST-1 mRNA expression. The same serine/threonine kinases activate the serine residues of the Smad2 linker region. Interestingly phosphorylation of the Thr220 residue and the mRNA expression of XT-1 were regulated by Jnk, p38 and PI3K, thus showing a correlation between the phosphorylation of the Thr220 and the initiation of GAG chain synthesis.

In VSMCs, TGF-β mediated Smad2 linker region phosphorylation of the serine residues occurs via the activation of Erk and p38 but not Jnk [19,20]. In our study thrombin phosphorylation of the serine residues required Erk and P38 but not Jnk. However a Jnk inhibitor abolished thrombin phosphorylation of theThr220 residue. Thrombin signals via transactivation of a protein tyrosine kinase and a serine/threonine kinase receptor [33]. In our earlier work we have shown that thrombin mediated Smad2 linker region phosphorylation partially occurs via transactivation of the EGFR [11]. There have been a number of studies showing that tyrosine kinase receptor agonists activate the Jnk pathway, leading to the phosphorylation of the Smad2/3 linker region. Hepatocyte growth factor treated RGM-1 cells activated the Jnk pathway, inducing phosphorylation of Smad2 linker region [34]. PDGF treated rat hepatic stellate cells rapidly increase the phosphorylation of Smad2/3 threonine residue which was inhibited in the presence of a Jnk antagonist. In human keratinocytes Jnk had no effect on thrombin mediated phosphorylation of the serine residues of the Smad2 linker region [35]. However in a rat model of liver injury, activation of Jnk was accompanied by phosphorylation of Smad2 Ser249 and 254 [36]. This shows that Jnk activation of the threonine residue of the Smad linker region may be specific for PTKR signalling in VSMCs.

The MAPK Erk regulated thrombin mediated phosphorylation of the Ser250 and Ser255 residues. In AKR-2B [37] and NIH/373 fibroblasts [38] TGF-β mediated phosphorylation of Ser245/250/255 is dependent on Erk. In CD4+ T helper cells Erk drives the phosphorylation of only the Ser255 residue [39]. A study from our lab showed that in BAECs, TGF-β activation of Erk leads to the phosphorylation of Ser245, Ser250 and Ser255 [27]. In TGF-β mediated phosphorylation of the Smad2 linker region in human VSMCs, Erk was involved in the phosphorylation of Ser245, Ser250 and Ser255 residues but not the phosphorylation of the Thr220 [19]. Interestingly, the Thr220 residue was not regulated by TGF-β mediated Erk phosphorylation in both BAECs and VSMCs and in our current thrombin model. Thrombin mediated mRNA expression of the two GAG genes (C4ST-1 and CHSY-1) involved in the elongation of GAG chains was regulated by thrombin. Thus these results are in agreement with our previous work that Erk driven pathways are associated with proteoglycan synthesis and in driving the expression of GAG synthesizing genes in VSMCs [19,20].

The present work identifies that thrombin phosphorylation of Ser245 and Ser255 is regulated by CDK. Similarly, in BAECs, CDK phosphorylates the Ser245 and Ser255 residues [27]. This shows that in these two cell models, CDK does not activate the Ser250 leading to PAI-1 expression or C4ST-1 and CHYS1 expression in VSMCs. Consistent with our results, CDK is not involved in the phosphorylation of the Thr220 residue in a melanoma model [40]. Direct activation of the TGFBR1 in VSMCs reveals that CDK is only involved in the phosphorylation of the Thr220 residue of the Smad2 linker region [19,21].

Interestingly, unlike the model proposed by our own data, TGF-β stimulation of CDK family members has a greater incidence for the phosphorylation of the Thr220 than the serine residues [41–43]. PI3K could act as an upstream mediator for CDK activation [44]. Thrombin mediated phosphorylation of the Smad2 linker region residues Ser245 and Ser255 occurs via PI3K and CDK however Thr220 is only dependent on PI3K activation. PI3K was also shown to participate in thrombin mediated mRNA expression of C4ST-1, CHSY-1 and XT-1. PDGF and thrombin are known to activate PI3K via Akt [45]. Data from our lab (unpublished) shows that PI3K is partially involved in thrombin mediated proteoglycan synthesis. VSMCs treated with thrombin increased the phosphorylation of the serine residues of Smad2 linker region, this was unaffected by Akt inhibitor [45]. These results demonstrate that, in VSMCs, thrombin regulation of the Smad2 linker serine residues does not involve the PI3K/Akt signalling but instead requires the PI3K/CDK pathways, correlating with the results showing that thrombin mediated linker region phosphorylation does not involve Akt [45].

Thrombin mediated mRNA expression of C4ST-1 and CHSY-1 occurs via transactivation of TGFBR1 and the EGFR [11]. The mRNA expression of the GAG synthesizing enzymes correlates with proteoglycan synthesis and GAG elongation [46]. TGF-β mediated GAG chain elongation is associated with Smad2 linker region phosphorylation. The CHSY-1 enzyme is directly involved in addition of repeated monosaccharide units on GAG chains, the sulfation enzymes C4ST-1 is involved in the sulfation of the GAG chain to provide docking sites for the addition of disaccharide units [47]. Thrombin can activate Erk, p38, PI3K and CDK to stimulate an increase in the mRNA expression of C4ST-1 and CHSY-1. Among the MAPKs involved in TGF-β signalling in VSMCs, Erk and p38 but not Jnk have been identified to modify GAG chains. In nucleus pulposus cells of invertebral discs, Erk participates in TGF-β regulation of CHSY-1 expression [48]. The data presented in this study is consistent with these two models.

Jnk did not regulate TGF-β mediated GAG gene [19] and GAG chain hyperelongation [20] in VSMCs. Thrombin phosphorylation of the Thr220 residue is regulated by Jnk, PI3K and p38. Interestingly thrombin mediated expression of the rate limiting genes involved in the elongation of GAG chains is not regulated by Jnk, however, the mRNA expression of the enzyme involved in the initiation of the GAG chains is regulated by Jnk, PI3K and p38. TGF-β mediated phosphorylation of the Smad2 Thr220 residue regulates XT-1 mRNA expression [19]. Knock down of XT-1 mRNA reduced GAG chain synthesis in cardiac fibroblasts [49]. Akt phosphorylation had no effect on TGF-β mediated GAG chain elongation however it played a role in proteoglycan synthesis [45]. In the current model of thrombin mediated GAG chain elongation as measured by the increase in the mRNA expression of C4ST-1 and CHSY-1, the PI3K/CDK not the PI3K/Akt pathway regulates GAG chain elongation, however the phosphorylation of the Thr220 residue was unaffected in presence of CDK inhibitor thus the Thr220 requires the activation of PI3K/Akt. Taken together the data indicates that phosphorylation of Thr220 residue regulates the expression of XT-1 gene.

5. Conclusions

We have shown that PAR-1 mediated mRNA expression of two GAG synthesizing genes associated with the elongation of GAG chains on biglycan correlated with the phosphorylation of the Smad2 linker region. However, PAR-1 mediated initiation GAG chain synthesis, as measured by the mRNA expression of XT-1, correlates to the phosphorylation of Thr220. Thrombin mediated GAG chain elongation requires pathways which activate Erk, p38 and PI3K/CDK but not Jnk, however the initiation of GAG chains require Jnk activation. These findings represent an example and a model of a concurrent signalling cascade which may provide a therapeutic target for human diseases, such as atherosclerosis, glomerulosclerosis and age related macular degeneration which have the underlying mechanism of lipid binding and entrapment by modified chondroitin sulfate/dermatan sulfate proteoglycans.

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Competing interests

There are no competing interests.

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