Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 815 (2005) 333–342
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantitative proteomic analysis of fibroblast nuclear proteins after stimulation with mitogen activated protein kinase inhibiting heparan sulfate

Johan Malmström[a], Jason Marchese[b], Peter Juhasz[b], Laurel Pukac[c], Morris Karnovsky[c], György Marko-Varga[d], Gunilla Westergren-Thorsson[a,*]

[a] *Department of Cell and Molecular Biology, C13, BMC, Lund University, S-221 84 Lund, Sweden*
[b] *Applied Biosystems, 500 Old Connecticut Path, Framingham, MA, USA*
[c] *Department of Pathology, Harvard Medical School, Boston, MA, USA*
[d] *Analytical Chemistry, University of Lund, Box 124, 221 00, Sweden*

Received 31 August 2004; accepted 29 November 2004

Abstract

Certain structures of heparan sulfate (HS) inhibit cell proliferation of fibroblasts. Whether this inhibition is dependent on inhibition of mitogenic signaling pathways or nuclear translocation of HS is unknown. In this study we investigated possible mechanism(s) and structural requirements by which antiproliferative glycosaminoglycans exert their effects on mitogen-activated protein kinase (MAP kinase) phosphorylation, a key intermediate in cell signaling, followed by quantitative proteomic analysis of nuclear proteins by stable isotope coded affinity tags, multidimensional chromatography and tandem mass spectrometry. Serum starved human lung fibroblasts were stimulated with serum, platelet derived growth factor (PDGF-BB) or epidermal growth factor (EGF) in the presence of structurally different glycosaminoglycans. Antiproliferative heparan sulfate with a high content of 2-*O*-sulfated iduronic acid (IdoA-2SO_4) and heavily sulfated glucosamine, and the structurally related glycosaminoglycan heparin inhibited significantly serum stimulated MAP kinase phosphorylation, by at least 80% when stimulated by serum and HS6. We hypothesized that the inhibition of the MAP kinase pathway will have effect in the nuclear proteome. Therefore an isotope coded affinity tag (ICAT™) reagent labeling of nuclear proteins and tandem mass spectrometry was applied, resulting in the identification and quantification of 206 proteins. Several nuclear proteins were found to be induced or repressed due to HS stimulation, where the repression EBNA-2 co-activator and the induction of PML protein were of special interest. These results show that heparan sulfate with high content of (IdoA-2SO_4) and heavily sulfated glucosamine specifically inhibits MAP kinase activation with a subsequent change in the nuclear proteome of the fibroblast.

Keywords: Cell growth; Fibroblast; Heparan sulfate; Mitogen-activated protein kinase (MAP kinase); Signal transduction

Abbreviations: bFGF, basic fibroblast growth factor; CS, chondroitin sulfate; DS, dermatan sulfate; DexS, dextran sulfate; EGF, epidermal growth factor; EMEM, Eagle's minimal essential medium; ERK, extracellular regulated kinase; F12-IT, Ham's F12 medium supplemented with insulin and transferrin; GAG, glycosaminoglycan; GlcNac, *N*-acetylated glucosamine; HS, heparan sulfate; IdoA, L-iduronic acid; MAP kinase, mitogen activiating protein kinase; MBP, myelin basic protein; PDGF, platelet derived growth factor; ICAT, isotope coded affinity tags; MS/MS, tandem mass spectrometry; MALDI, matrix assisted laser desorption ionization; MS, mass spectrometry; TOF, time of flight; CAN, acetonitrile; SCX, strong cationic exchange; LC, liquid chromatography

* Corresponding author. Tel.: +46 46 222 33 14; fax: +46 46 222 31 28.
E-mail address: gunilla.westergren-thorsson@medkem.lu.se (G. Westergren-Thorsson).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.11.054

1. Introduction

The extracellular matrix (ECM) has an important role in influencing cell behavior through several different pathways either by directly interacting with cell surface receptors and subsequent activation of signaling pathways or through activating/inhibiting growth factors [1]. One group of ECM molecules that interact with both growth factor ligands and their receptors are the heparan sulfate (HS) proteoglycans [2]. The HS proteoglycans consist of HS chains attached to different core proteins, which are integrated into the plasma membrane of many different cell types. The major groups of HS proteoglycans are the transmembrane proteoglycan (the syndecans) [3], the glycosylphosphatidylinositol-anchored proteoglycan (the glypican) and the matrix associated proteoglycan (e.g. perlecan). The syndecans are the major source of cellular heparan sulfate glycosaminoglycans and are important for regulating proteases in a wound environment [4]. The HS chains are polymers of the disaccharide glucuronic acid β1,4-*N*-acetylglucosamine α1,4- and the HS-protein interactions are dependent on certain sequences of the HS chains [5–7]. These polymers are built up and divided into block regions of repeats of *N*-acetyl glucosamine or *N*-sulfated glucosamine. The *N*-sulfated regions are often further modified by epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA) followed by 2-*O*-sulfation and 6-*O*-sulfation on the *N*-sulfated glucosamine and occasionally 3-*O*-sulfation occurs on specific sites. The block regions are enriched in structures such as IdoA, 2-*O*-sulfation and 6-*O*-sulfation. HS interact with a number of different molecules such as growth factors [8] proteases [4], ECM components [5], and chemokines [9] and thereby modifying their availability and activity.

HS proteoglycan metabolism is very rapid with a half-life of 5–20 h [10]. This means that the HS proteoglycans can undergo several structural changes during one cell cycle. The overall structure of the HS proteoglycans are modified by for example transformation [11] and aging [8] and their structure often reflect the metabolic state of the cell. The structural modifications alter the binding properties to different growth factors as well as cellular behavior. It has been shown in a previous study that HS rich in IdoA- and sulfate increases the mRNA levels for the PDGFα receptor and the number of binding sites for PDGF [12]. The same species of HS also inhibit proliferation of lung fibroblast more potently than heparin [12,13]. HS poor in sulfate and IdoA have no effect on either the level of PDGF receptors or on proliferation indicating that the structural specificity is of importance for the above described effects [12,13]. To further explain the mechanism behind these results, a number of binding and endocytosis studies have been performed [14–16]. Arroyo-Yanguas et al. found that HS with antiproliferative capacity bind to the cell surface with one low affinity site and a high affinity site. The low affinity site is probably associated with endocytosis and the high affinity with signal transduction pathways. It has also been shown that antiproliferative HS can be localized in the nucleus, indicating that HS can directly affect transcription [17–19].

In this study we investigated if the binding of antiproliferative HS could affect cell proliferation by interfering with the signal transduction cascade associated with cell proliferation in fibroblasts. The main signal transduction pathway, which is quickly activated due to a mitogenic signal, is the mitogen activated protein kinase (MAP kinase) pathway. Ras recruits Raf, which initiates the MEK and ERK phosphorylation cascade [20]. ERK consist of two isoforms ERK 1 and ERK 2, where phosphorylation of ERK 2 has specifically been associated with cell proliferation [20]. It has been shown earlier that heparin can inhibit MAP kinase activity in smooth muscle cells [21,22], possibly due to a decrease in the signaling of protein kinase C [23–25]. In this investigation we studied the effect of the glycosaminoglycans (GAG) on MAP kinase signaling and the structural requirement for obtaining maximal effects. We found that only antiproliferative HS and heparin inhibited activation and phosphorylation of MAP kinase and that the high affinity-binding site of heparan sulfate seems to be important in mediating these effects. By quantitative proteomic analysis by isotope coded affinity tags (ICAT^{TM}) and tandem mass spectrometry a subsequent alteration in the nuclear proteome was investigated.

2. Materials and methods

2.1. Materials

Heparin (Hep) M_r 20 000 from beef lung was a product from Glaxo, Greenford, U.K. The HS (HS2, HS6) used were prepared from beef lung by methods described previously [26,27]. They were further purified by gel chromatography on Superose 6 under dissociative conditions before use, for more details see [13]. Final preparation step was separation on a Sepharose 6 column under dissociative conditions to remove cytoactive components bound to the HS. The procedure for the determination of total hexosamine, uronic acid and sulfate content has earlier been described [13,14,26–28]. The DS was obtained and characterized by methods described previously. The CS M_r 20 000 was from bovine nasal cartilage and was a gift from the Chicago standard collection. GAGs were released from their parent PGs (when applicable) by extensive proteolysis using papain. To remove possible contamination by HS in DS preparations, they were treated with a weak acid. Chemical data are summarized in Table 1 [26,27,29].

Anti phosphotyrosine monoclonal antibodies made in rabbit were bought from Transduction Laboratories, Lexington, USA. Biotinylated anti-rabbit IgG (H + L) (affinity purified) and the vectastain elite, ABC kit were bought from Vector Laboratories, Burlingame, CA, USA. BCA, Protein Assay Reagent Kit, was from Pierce, Rockford, Illinois, USA. Dextran sulfate (DexS) M_r 5000, anti ERK polyclonal antibodies made in rabbit and myelin basic protein (MBP) were all obtained from Sigma, St. Louis, MO, USA. Immune-Blot^{R}

Table 1
Chemical data for sulfated glycosaminoglycans used

Sample	*O*-SO_3	*N*-SO_3	IdoA	IdoA-*O*-SO_3	M_r
	HexN (mole/mole)	HexN (mole/mole)	HexA (%)	HexA (%)	
HS2	0.30	0.26	30	10	20
HS6	0.91	0.72	65	60	20
Hep	1.91	0.65	80	75	20
DS	1.16	n.a.	90	20	30
CS	1.0	0	0	0	20
DexS	0	0	0	0	5

Hex: hexosamine; HexA: hexuronic acid; n.a.: not applicable; *O*-SO_3: ester sulfate; *N*-SO_3: *N*-sulfamate. Relative molecular mass (M_r) was determined by gel chromatography on Superose 6 calibrated with HS-species of known molecular mass (determined by light scattering).

PVDF membrane for blotting (0.2 μm) was purchased from Amhersham Pharmacia Biotech AB, Uppsala, Sweden. Recombinant human PDGF-BB was from R&D System, Oxon, U.K. and recombinant human EGF from Genezyme, Cambridge, MA, USA. [γ-^{32}]ATP and autoradiography film were bought from New England Nuclear, Boston, MA, USA.

2.2. Cell culture

Fibroblasts were established from human lung embryonic tissue and grown in 25 cm^2 dishes in Eagle's minimal essential medium (EMEM) supplemented with 10% new born calf serum and 1% glutamine, at 37 °C in a humidified incubator with 5% CO_2 and 95% air atmosphere. Experiments were performed on cells between passage 5 and 20. Cells were regularly checked for mycoplasma with a GEN-PROBE Rapid Detection System (Gen Probe, San Diego, CA, USA).

2.3. Experimental design

Quiescent cells were obtained by growing semi-confluent cells in medium with 0–0.4% new born calf serum for 48 h. The quiescent cells were then stimulated by medium supplemented with either 10% new born calf serum, PDGF (10 ng/ml) or EGF (20 ng/ml) for time periods ranging between 10 and 40 min [28]. GAGs were added in concentrations between 0.1 and 100 μg/ml in the presence of medium containing 10% serum. Negative controls were quiescent cultures grown in serum free medium. For isolation of cell extracts following stimulation, the cells were washed four times in cold PBS and then detergent extracted at 4 °C in 100 μl RIPA per six-plate well (50 mmol/l Tris–Cl (pH 7.2), 150 mmol/l NaCl, 40 mmol/l NaF, 5 mmol/l EDTA, 5 mmol/l EGTA, 1 mmol/l NaOV, 1% NP40, 0.1% Na deoxycholate, 0.1% SDS, 0.042 mmol/l leupeptin, 0.003 mmol/l aprotinin and 1 mmol/l PMSF). After centrifugation the cell supernatant was aliquoted and then stored in −70 °C until used.

2.4. Analytical methods

2.4.1. Protein quantification

The amount of protein in all extracts was determined by BCA Protein Assay Reagent Kit Pierce, Rockford, Illinois and analyzed by spectrophotometer at 595 nm according to the manufactures instruction.

2.4.2. Western blot

The samples were separated by 8–10% SDS-polyacrylamide gel for 50 min 200 V (Mini-PROTEAN II Electrophoresis Cell, Biorad, Hercules, CA, USA). The proteins were transferred from the gel to a PVDF membrane by semidry electroblotting for 50 min in 25 V (Trans-blot SD Semi-Dry Electrophoretic Transfer Cell, Biorad, Hercules, CA, USA). The PVDF membranes were incubated with affinity purified rabbit monoclonal antibody for phosphotyrosine or rabbit polyclonal antibody for ERK and washed in Tris buffer saline (TBS) containing 1% BSA and 0.1% Tween. The membranes were incubated with biotinylated goat anti-rabbit and washed with 0.1% Tween TBS, followed by a second wash in TBS. Immunoreactivity was visualized by vectastain ABC detection system all according to ref. [22]. The membranes were scanned and analyzed with image analyzer and gel pro analyzer.

2.4.3. Activity analysis

The same samples as used above were separated on a 8% SDS-polyacrylamide gel containing myelin basic protein (7.55 μmol/l), a substrate for MAP kinase described previously. The gel was incubated with [γ-^{32}]ATP for 2 h and then washed carefully to remove unspecific radioactivity. The radioactivity was visualized by using autoradiography.

2.5. Purification of nuclei

The nuclei were purified according from a protocol described in Jung et al. [31], with some minor changes. Briefly, the cells were harvested in buffer A (60 mmol/l KCl, 15 mmol/l NaCl, 0.15 mmol/l Spermine, 0.5 mmol/l Spermidine, 15 mmol/l Hepes and 14 mmol/l Mercaptoethanol) supplemented with 0.2% (v/v) Nonidet p40, aprotinin (0.15 μmol/l), leupeptin (2.1 μmol/l), pepstatin A (1 μmol/l) and PMSF (1 μmol/ml) and 0.3 mol/l sucrose. The cell suspension was then homogenized with 40 strokes at 2000 rpm (Labortechnik, Berlin, Germany) and the homogenate was filtered with 100 μm filter paper (Schleicher&Schuell, Dassel, Germany). The nuclei were counted in a Bürker chamber and the purity was assessed and protein content was determined by Bradford protein reagent kit (Pierce, Rockford, IL, USA).

2.6. Labeling with the acid-cleavable ICAT™ reagent and isolation of cysteine containing peptides

Fibroblast nuclei were lysed in a buffer containing 6 mol/l Guanidium–HCl (pH 8.5), 1% Triton X-100, 50 mmol/l

Tris–HCl [30]. Starting with 500 μg for control and HS stimulated, each sample was reduced by adding 10 μl of 50 mmol/l Tris(2-carboxyethyl)phosphine (TCEP) and boiled for 10 min. The samples were allowed to cool and the acid cleavable ICAT™ reagent (all the materials in this section are from the ICAT™ reagent kit Applied Biosystems, Framingham, MA, USA) was added: the light reagent to the control and heavy reagent containing nine ^{13}C to the HS treated sample [31,32]. Alkylation was allowed to complete for 2 h, at 37 °C. The two samples are combined and acetone precipitated in order to remove the guanidium–HCl and the unreacted ICAT™ reagent. The pellet was dissolved in a buffer consisting of 50 mmol/l Tris (pH 8.5), 5 mmol/l $CaCl_2$, and 10% acetonitrile (ACN). Trypsin was added at a 1:40 enzyme/substrate ratio in two additions once at the start of the digestion and once more, 2 h later. Digestion was completed at 37 °C overnight.

Following digestion the sample was diluted into 25% ACN, pH 3.0 in order to reduce the buffer concentration below 10 mmol/l. The resulting peptide mixture was injected to a PolyLC (4.6 × 100 mm Polysulfoethyl A) strong cationic exchange (SCX) column on Vision workstation (Applied Biosytems, Framingham, MA) at a flow rate of 1 mL/min using a binding buffer (buffer A) 10 mmol/l KH_2PO_4, 25% ACN, pH 3 and an elution buffer (buffer B) of 350 mmol/l KCL, 10 mmol/l KH_2PO_4, 25% ACN, pH 3. The gradient was 0% B to 10% B in 2 min, 20% B in 15 min, 45% B in 3 min, 100% B in 10 min and an additional 8 min hold at 100% B. Twenty-three SCX fractions of 1.5 ml volume were collected.

The pH was adjusted for each SCX fraction to 7.2 with 10× PBS buffer (pH 10) and injected to the avidin affinity column as prescribed by the manufacturer (Applied Biosystems, Framingham, MA). Cysteine-containing peptides bound to the avidin column were washed three times: 1 ml of Wash 1 and 1 ml of Wash 2 and 1 ml of de-ionized water. Elution of the cysteine-containing peptides was preformed with 800 μl of avidin elution buffer. The samples were then concentrated by a speed-vac to dry, cleaved with the ICAT™ cleaving reagent containing 95% TFA and concentrated again to remove the cleaving reagent. The samples were then taken up in 2% ACN, 0.1% TFA for re-injection for a nanoHPLC mass spectrometry (MS) and MS/MS analysis.

2.7. Protein identification and quantification by LC–MS and MS/MS

Avidin purified SCX fractions were subjected to matrix assisted laser desoprtion (MALDI) HPLC–MS and MS/MS analysis. The fractions were injected in the HPLC loading buffer onto a 100 μm × 15 mm C_{18} Magic column (Michrom Bioresources, CA) using a CapTrap pre-column (Microm Bioresources). Mobile phase A was 2% ACN, 0.1% TFA, mobile phase B was 85% ACN, 5% *n*-PrOH, 10% water, 0.1% TFA. HPLC elution was carried out at a 1-μl/min flow rate on an Ultimate nanoHPLC workstation (Dionex-LC Packings, Hercules, CA). The HPLC elution from the column was collected at 20-s intervals on the MALDI plate using a Probot fraction collector (Dionex-LC Packings). 48-min HPLC elution was collected to the MALDI plates as an array of 12 × 12 sample spots. The HPLC eluent was mixed with the MALDI matrix (7.5 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 60:40 acetonitrile–water containing 0.15 mg/ml dibasic ammonium-citrate) through a mixing tee (Upchurch, WA) at a flow rate of 2-μl/min. The most abundant, middle-fractions of the SCX separation were spotted onto two plates using a 90-min HPLC gradient.

MALDI plates were analyzed in automated mode on the AB4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). First the MS spectra were collected from the entire HPLC run. Then, using an in-house developed program, MS/MS precursors were selected by applying an exclusion algorithm to eliminate: (a) redundant precursors carrying over multiple HPLC fractions, and (b) using only the more abundant members of peptide pairs for MS/MS analysis. MS/MS spectra were acquired using up to 2500 laser shots/precursor unless the pre-defined signal-to-noise level in the MS/MS acquisitions was achieved sooner. The MS/MS data were submitted for database searching as a batch to Mascot (http://www.matrixscience.com) through its automation interface of Mascot (Mascot Daemon). The non-redundant NCBI protein database was used. A detailed description acceptance criteria for the database searching results will described elsewhere. In brief, tryptic peptides containing arginine residues were accepted at a Mascot score >20, peptides not containing arginine at a Mascot score >25. Peptides with less-than-significant Mascot score were thoroughly inspected, considering the correlation of peptide basicity with SCX fraction numbers, presence of characteristic high-energy collision induced dissociation (CID) fragments, and accurate mass measurements through internal calibration of the MS spectra using the masses of confidently identified peptides as internal mass references.

2.8. Statistical method

Mean ± standard errors of the mean (S.E.M) were calculated. Student's *t*-test was used to evaluate the differences of the means between groups.

3. Results

3.1. Structural specificity of the GAGs in the inhibition of MAP kinase phosphorylation

To determine the structural specificity of the response on phosphorylation of MAP kinase, different forms of GAGs were used at a concentration of 100 μg/ml together in MEM supplemented with 10% fetal calf serum (Fig. 1). Heparan sulfate high in iduronic acid and sulfate (HS6) and the structurally similar heparin were able to inhibit

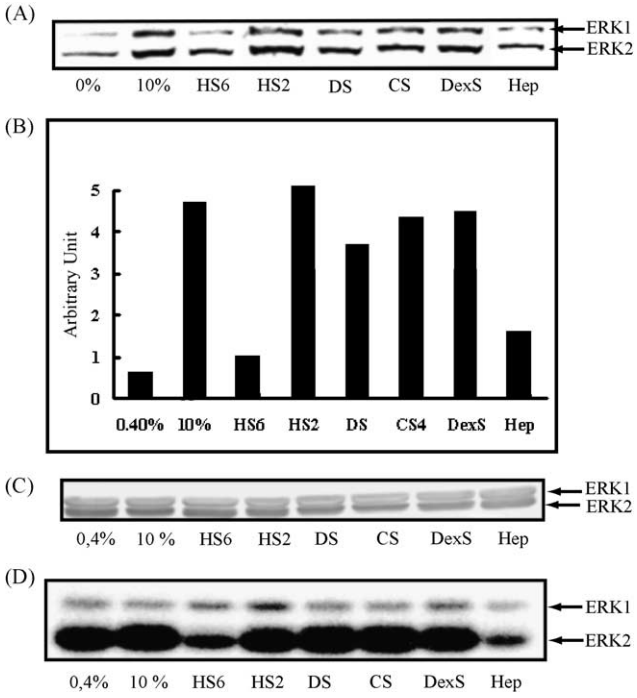

Fig. 1. Effect of different glycosaminoglycans on the phosphorylation of mitogen-activated protein kinase (MAP kinase). (A) Serum-starved embryonic lung fibroblasts were incubated with 100 μg/ml of various glycosaminoglycans for 30 min. Negative control was medium containing 0–0.4% serum and positive control was medium with 10% serum. Western analysis was performed using an antiphospho-ERK antibody. Arrowheads indicate the position of the 44 000 rel. mol. mass units (ERK 1) and the 42 000 rel. mol. mass units (ERK 2) MAP kinase isoforms. The blot shows a representative experiment out of three. All samples were normalized to the same protein level before samples were applied on the gel. (B) The intensity of the 44 000 rel. mol. mass units and the 42 000 rel. mol. mass units bands from the Western blot in (A) was measured. The sum of the two ERK isoforms is shown. (C) To role out the possibility that the different band intensities were not due to a transcriptional or translational effect, the cells were treated the same way as above and Western blot was performed by using an anti ERK antibody. The picture shown is a representative blot from the same lysate as in (A) and the bands shown are the only ones present. (D) In-gel kinase assay to determine MAP kinase activity as detected by MAP kinase phosphorylation of myelin basic protein substrate incorporated in polyacrylamide gel. The arrows indicate the position of the activated MAP kinase isoforms, the bands of ERK 1 and ERK 2. Negative control was medium containing 0.4% serum and positive control was medium with 10% serum. Abbreviations used for the substances tested are IdoA- and sulfate rich HS (HS6), IdoA- and sulfate poor HS (HS2), dermatan sulfate (DS), chondroitin sulfate (CS), dextran sulfate (DexS) and heparin (Hep).

the phosphorylation of the 44 000 and 42 000 rel. mol. mass units MAP kinase isoforms determined by anti-phospho ERK antibodies. The inhibition by HS6 was 74% ± 0.6% ($p < 0.05$) and 73% ± 5% ($p < 0.001$) for ERK 1 and ERK 2, respectively (Fig. 1A shows a representative experiment). Dermatan sulfate (DS) and dextran sulfate inhibited MAP kinase phosphorylation by less than 30% of control in both cases, which was not significant (Fig. 1A and B). Western blot performed with ERK antibodies showed that inhibition of phosphorylation of MAP kinase was not due a transcriptional or translational variation in the amount of the MAP kinase proteins (Fig. 1C). Furthermore, an in gel kinase assays with myelin basic protein as a substrate were further used to directly asses MAP kinase activity in the same samples as above. This experiment confirmed that changes in tyrosine phosphorylation detected by Western blot correlated with decrease in MAP kinase activity by HS6 and heparin (Fig. 1D). The same amount of protein was applied in all lanes of every sample for both Western blot and in-gel kinase assays.

3.2. Heparan sulfate high in IdoA and sulfate (HS6) down-regulates phosphorylation of MAP kinase in a dose- and time-dependent manner

The time- and dose response of the effect of HS6 on MAP kinase phosphorylation was further studied. Fibroblast cells were incubated as above and treated with 10% serum and HS6 for time periods of 10 up to 40 min. HS6 inhibition of ERK phosphorylation by 50% after 10 min, 55% after 20 min and 75% after 30 and 40 min (Fig. 2).

To investigate the signaling pathway specificity of MAP kinase activation, cultures were incubated with HS6 and either PDGF-BB, EGF or 10% FCS. For the dose response studies the cells were stimulated by 10% serum, PDGF-BB (10 ng/ml) or EGF (20 ng/ml) together with HS6 in doses between 0.1, 1, 10 and 100 μg/ml for 30 min. All three mitogens activated MAP kinase, EGF stimulation resulted in the highest activation with a 22-fold increase of MAP kinase phosphorylation (data not shown). Inhibition of serum stimulated ERK phosphorylation reached a maximum at a concentration of 1 μg/ml HS6 for ERK 1 (44 kDa) and at 10 μg/ml HS6 for ERK 2 (42 000 rel. mass units) (Fig. 3). The inhibition seen at these concentrations were 74% ± 1.4% ($p < 0.05$) and 74% ± 0.6% ($p < 0.05$) for ERK 1, respectively and 67 + 1.5% ($p < 0.001$) and 67% ± 4.4% ($p < 0.001$) for ERK 2 (Fig. 3A). HS6 was not able to significantly down regulate the stimulatory response of PDGF-BB (Fig. 3B). The repression of EGF stimulated ERK phosphorylation was weaker compared to serum stimulation, reaching significant repression only at concentrations of 10 and 100 μg/ml HS6 for both ERK 1 and ERK 2. The inhibition seen at this concentrations was 27% ± 0.6% ($p < 0.05$) for ERK 1 respectively 34% ± 2% ($p < 0.05$) and 30% ± 4.7% ($p < 0.05$) for ERK 2 (Fig. 3C).

3.3. High affinity site of binding of heparan sulfate (HS6) may be necessary for inhibition of MAP kinase phosphorylation

HS6 binds to one high affinity site and to one low affinity site on the cell surface of fibroblasts [14]. As shown previously DexS, is without significant effect on cell proliferation [13] and MAP kinase activation (Fig. 1), DexS can however block HS6 binding to the cell surface and abolish the antiproliferative effects of HS6. It was therefore of interest to see if the binding to the cell surface was necessary for inactivation of MAP kinase. Fibroblasts were incubated with 10% serum

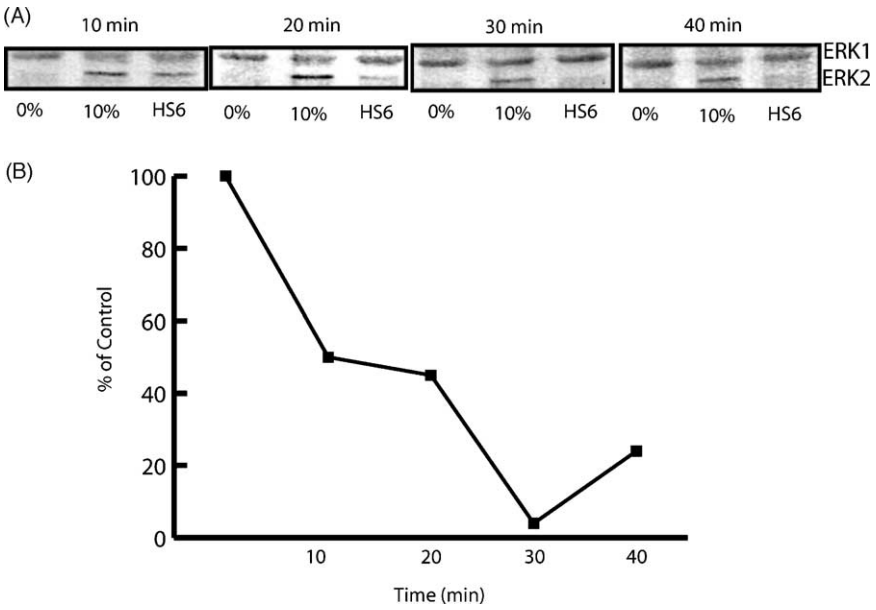

Fig. 2. Time dependent inhibition of MAP kinase phosphorylation by IdoA- and sulfate rich HS (HS6). (A) Serum-starved embryonic lung fibroblasts were incubated with of 100 μg/ml of HS6 for 10–40 min. Negative control was medium containing 0.4% serum and positive control was medium with 10% serum. Western analysis was performed using an antiphosphotyrosine antibody. Arrowheads indicate the position of the ERK 1 and ERK 2 MAP kinase isoforms. The blot shows a representative experiment out of two. All samples were normalized to the same protein level before samples were applied on the gel. (B) The intensity of ERK 1 and ERK 2 from the Westerns blot in (A) were measured and the sum of the two bands is shown. Relative factor of control was obtained by dividing the bands from cultures treated with GAG by the bands from cultures incubated with 10% serum alone.

together with HS6 and DexS in various concentrations. Ten μg/ml of HS6 was used which is the concentration where maximum inhibitory effect of phosphorylation for both ERK 1 and ERK 2 was seen (Fig. 3). At 1 mg/ml of dextran sulfate the inhibition of MAP kinase phosphorylation by HS6 was reduced by 33.3% ± 1.6% ($p < 0.05$) (Fig. 4), but the inhibition of MAP kinase activation was still significant for both ERK 1 ($p < 0.05$) and ERK 2 ($p < 0.01$). When the dextran sulfate concentration was increased to 10 μg/ml which is twice as much dextran sulfate as HS6 on a molar basis, the down regulation of the MAP kinase phosphorylation was reduced by 66.6% ± 21% (Fig. 4). Twenty μg of DexS resulted in a 33% ± 6.5% reduction of the inhibitory effect of HS6 on MAP kinase phosphorylation. When higher doses of DexS (10 μg/ml and 20 μg/ml) were used the significant inhibitory effects from HS6 on MAP kinase inhibition disappeared (Fig. 4).

3.4. ICAT labeling and tandem mass spectrometry for identification and quantification of nuclear proteins

MAP kinase has several downstream effects that subsequently results in altered gene transcription and protein expression profiles. In order to study whether the inhibition of MAP kinase resulted in protein regulations within the fibroblast nucleus that can elucidate the pathways that are affected by the inhibition of MAP kinase by HS, we used isotope coded affinity tags (ICAT), multidimensional chromatography and tandem mass spectrometry (MS/MS), described previously. The ICAT labeling enables quantitative analysis of the separated peptides when analyzed in MS mode and these quantified peptides were then identified in MS/MS mode and database search against SWISSPROT. For a typical MS and MS/MS resulting in protein quantification and identification, in this case PML-1, see Fig. 5. Using this approach 206 unique proteins were identified and quantified and divided into groups (Fig. 6). From the list of identified proteins, ten proteins were of particular interest selected with the two following criteria; (1) known or predicted nuclear localization and (2) a regulation factor of >0.75 or <1.25 (Table 2). Of the 10 selected proteins, in eight of the proteins the expression was induced and in two of the proteins the expression repressed (Table 2). The two repressed proteins were a RNA helicases and EBNA-2 co-activator. The proteins were four heterogeneous ribonucleoproteins, one splicing factor and PML-1 protein. In addition two less characterized proteins, programmed cell death 8 and a myeloid-associated differentiation marker, were identified (Table 2).

4. Discussion

Heparin and heparan sulfates are potent inhibitors of human fibroblast proliferation. The aim with this investigation was to investigate if the inhibition of cell proliferation, by certain structures of glycosaminoglycans, was mediated through inhibition of MAP kinase activity and the subsequent effects in the nuclear proteome.

Several different kinds of glycosaminoglycans, some which inhibit cell proliferation and some which do not, were

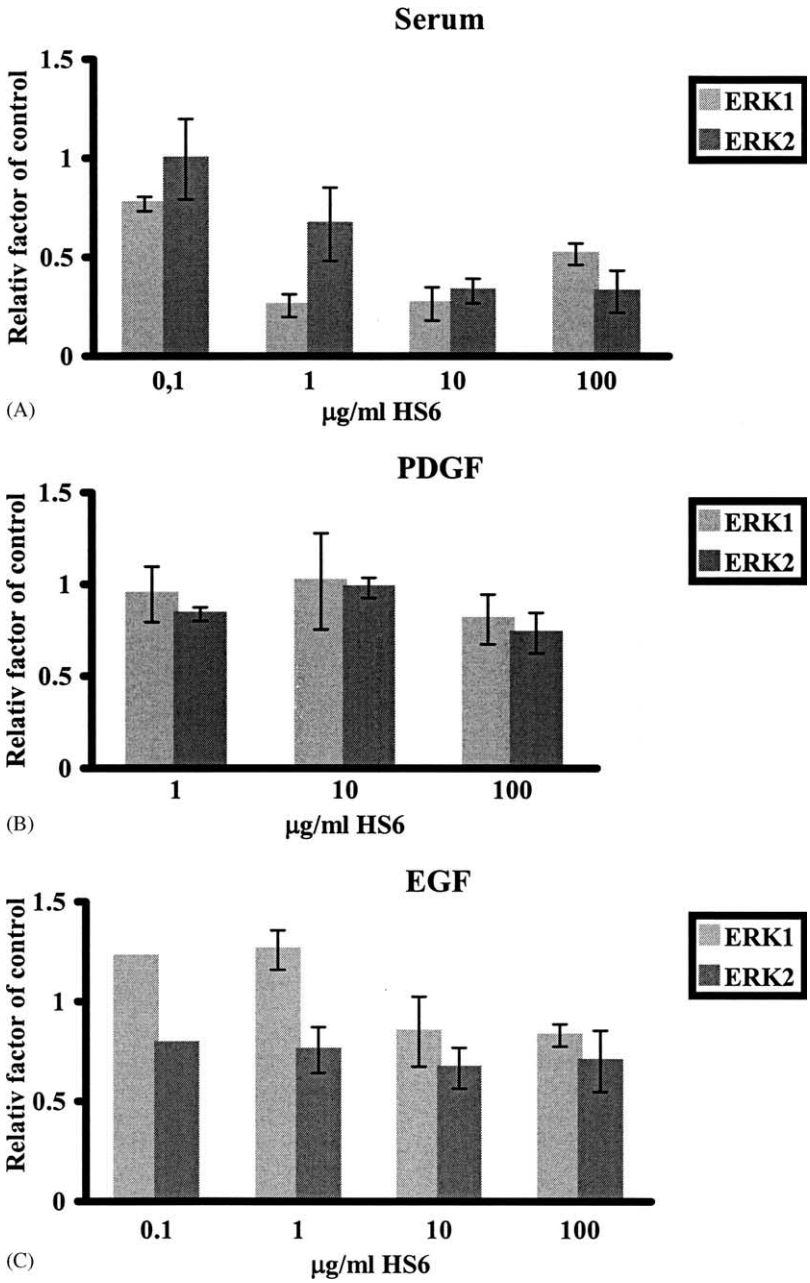

Fig. 3. Dose response curve for inhibition of MAP kinase phosphorylation by the IdoA- and sulfate rich HS (HS6) in cultures stimulated by serum (A), PDGF-BB (B) or EGF (C). Lung fibroblasts were incubated with HS6 in a dose of 0.1–100 μg/ml for 30 min along with 10% serum, PDGF-BB (10 ng/ml) or EGF (50 ng/ml). Positive control was medium with 10% serum, PDGF-BB or EGF. The intensity of the ERK 1 and the ERK 2 bands from the Westerns blot were measured. The graphs display the relative factor of control, on the *y*-axis, which is the measured ERK intensity from cultures treated with HS6 and serum, PDGF-BB or EGF divided by the measured ERK intensity for the positive control. Values in the figures are $x \pm$ S.E.M., $n = 5$.

used. HS2, CS nor DexS, which do not inhibit cell proliferation or antiproliferative DS had any significant effect on ERK activation, whereas antiproliferative HS (HS6) and heparin had strong anti-ERK effects. Arroyo-Yanguas et al. [14] showed previously that antiproliferative DS do not bind very well to the cell surface, whereas HS does, suggesting that the inhibitory effects of MAP kinase are mediated through interactions on the cell surface. Both HS6 and heparin have high contents of both sulfation and IdoA, whereas HS2, with lower amounts of sulfation and IdoA was unable to affect MAP kinase activity. There is thus a positive correlation between high amount of sulfation and IdoA and inhibition of both cell proliferation and inhibition of MAP kinase activity.

ERK 1 and ERK 2 are activated by many different stimuli. Serum, EGF and PDGF all activate MAP kinase through ligand/receptor interaction, which propagates a signal down to the nucleus. HS inhibition of MAP kinase activation was observed after stimulation with serum and

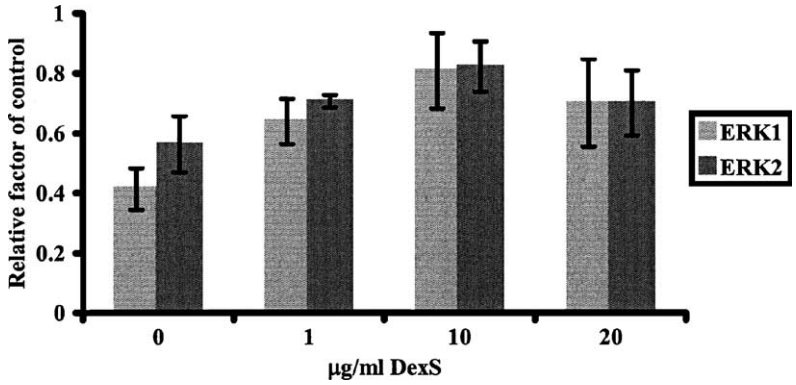

Fig. 4. Dextran sulfate reduces the HS6 inhibition of MAP kinase phosphorylation by competing for binding to the high affinity site. Lung fibroblasts were incubated with 10 mg/ml HS6 and 10% serum alone or together with 1 mg/ml, 10 mg/ml or 20 mg/ml of dextran sulfate (DexS). Cells were also incubated with 10% serum and DexS 20 mg/ml. Positive control were cells incubated with only 10% serum alone. The intensity of the ERK 1 and the ERK 2 bands from the Westerns blot were measured. Relative changes are shown as the ratio of the bands from cultures treated with HS6 and serum alone or together with various concentrations of DexS divided by bands from the positive control. Values in the figures are $x \pm$ S.E.M., $n = 3$.

EGF but not after stimulation with PDGF. However, the inhibition of the EGF induced stimulation of MAP kinase was less potent compared to the inhibition MAP kinase phosphorylation with serum. This is in accordance with previous studies showing that heparin is unable to inhibit MAP kinase stimulation from bFGF and PDGF [33] and EGF in smooth muscle cells [22]. It has been suggested that heparin only inhibits MAP kinase activation elicited by ligands of seven transmembrane domain receptors and heterotrimeric G-proteins [34] or by PKC [24]. Because of the different

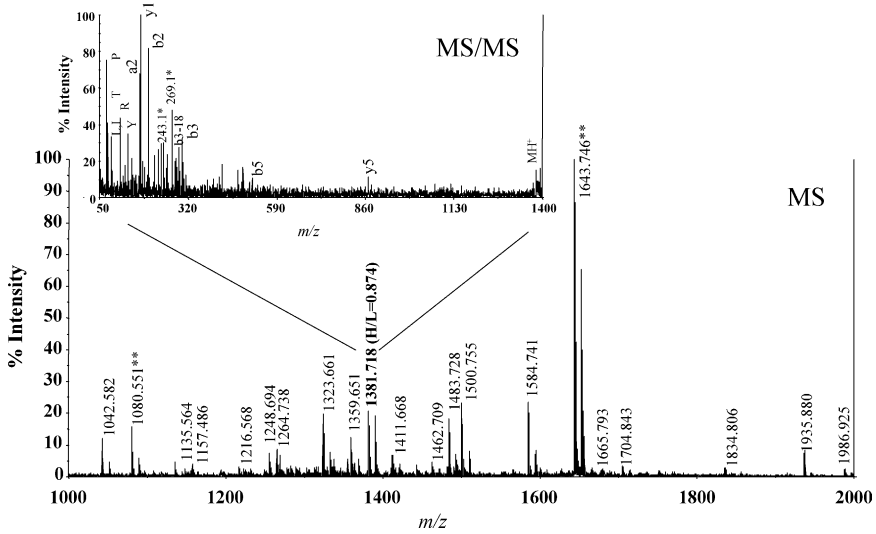

Fig. 5. Quantifying and identifying PML-1 protein, through the peptide sequence: TPTLTSIYCR. One MS spectrum from HPLC fraction from strong cation exchange fraction 8. Pairs of ICAT labeled peptides are clearly visible where masses are displayed for the light variants only. The mass difference between a pair single-cysteine containing peptide is 9.030 Da. The average ratio of the components from the HS treated sample (labeled with the heavy reagent) to the control sample (labeled with the light reagent) is around 0.5 with a standard deviation of 0.14 (as determined from the 1000 ICAT pairs in SCX fraction 8). Once normalized to the average, the pair at 1381/1390 occurs differentially regulated. The MS/MS spectrum of the precursor 1381.7 is shown in the inset. The Mascot score associated wit this spectrum is barely significant, although some features in the MS/MS spectrum – namely the unusually abundant monoamino acid fragment from T suggesting the presence of multiple threonines, strong y1, a2 and b2 fragments – give added credibility to this identification. (Fragments with * are signatures of the ICAT labeled cysteine residue). Database searching with Mascot used 100 ppm tolerance on the mass of the precursor. The presence of components with high-confidence identifications in the MS spectrum can be utilized for improving the mass accuracy of precursor masses to as tight as a 10-ppm tolerance. Peaks in the MS spectrum marked by ** represent known components with high Mascot scores. These two components (four theoretically known masses if both heavy and light labeled components are considered) were used as internal reference masses and the MS spectrum was re-calibrated. The mass labels in the figure correspond to the values after internal calibration. The theoretical mass of the light-ICAT labeled TPTLTSIYCR sequence is 1381.715 Da (as MH+): the experimentally determined 1381.718 value has an error of 2 ppm. Checking the SWISSPROT (ver. 10.25.2002) protein database, only 20 tryptic peptides from human proteins were found in a ±5 ppm mass window around 1381.718 Da. Out of 20 peptides, only four unique sequences were found with a single cysteine residue in it, one of them being TPTLTSIYCR. The other three sequences were, however, were fully incompatible with the MS/MS spectrum shown above.

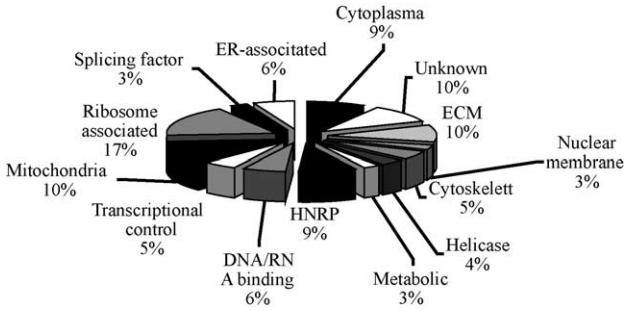

Fig. 6. Functional grouping of identified proteins. The proteins were divided up into functional groups. The pie graph shows the distribution of proteins related to cellular localization and function.

pathways used by heterotrimeric G-proteins to activate MAP kinase it is difficult to predict the molecular mechanisms behind this inhibitory effect by heparin and heparan sulfate.

Arroyo-Yanguas et al. [14] showed that the HS chains bind specifically to the cell surface with one high and one low affinity site. We hypothesize that the low affinity site is connected to the endocytosis pathway leading to the subsequent enrichment of certain HS structures in or around the nucleus [18,35], and that the high affinity site is associated with signaling pathways like MAP kinase. The high affinity site, which is saturated at 1 μg/ml HS6 is the dose where inhibition of MAP kinase is inhibited but cell proliferation is unaffected. By co-incubate HS chains with an excess of dextran sulfate, that occupies the same binding site as HS, we wanted to block the high affinity site to see if the high affinity site is responsible for the inhibition of MAP kinase phosphorylation. After incubation with 1 μg/ml DexS, HS6 is still able to significantly down regulate MAP kinase activation. However when increasing the doses of DexS the significant effects disappeared indicating that the high affinity site is important for the inhibition of MAP kinase. We have here indications that the high affinity site is important for inhibition of MAP kinase phosphorylation, whereas inhibition of cell proliferation is dependent on both the low and the high affinity binding sites.

MAP kinase has previously been shown to affect several immediate early response genes. In order to study whether HS induced expression changes of the nuclear proteome, we employed ICAT, multidimensional chromatography and tandem mass spectrometry to study protein expression in the nucleus after HS stimulation. We found that HS induced the expression of four heterogeneous nuclear ribonuclear proteins (hnRNPs). Additionally, of especial interest were the repression of the EBNA-2 coactivator and the induction of promyeolocytic leukemia protein-1 (PML-1). EBNA-2 is one of the earliest virally encoded nuclear proteins expressed after an Epstein-barr viral infection and participates in driving resting cells to enter the cell cycle [36]. EBNA-2 functions as a transactivator for proteins like c-myc and interacts with DNA through the mediation of the cellular transcription factor [37]. The PML protein is the signature protein of the PML nuclear bodies, which participate in growth control, transformation, suppression, apoptosis and Ras induced senescence [38]. Overexpression of the PML protein results in G1 arrest and subsequent disruption of PML nuclear bodies by mutations in PML correlates to the loss of this growth suppression. Furthermore, it has been shown that PML suppresses the protein levels of cyclin D1. In this study, it was observed that antiproliferative HS reduced the expression of a coactivator to the cell cycle activator EBNA-2 and induced the expression of the cell cycle repressor PML [39–43]. These data indicate that the antiproliferative effects of HS are mediated through inhibition of MAP-kinase signaling pathway resulting in specific regulation of proteins involved in cell cycle control. However, further characterization of the functional role of the EBNA-2 co-activator and the PML protein in cell cycle control and their association to the MAP kinase pathway is required before a detailed explanation of the mechanism behind the antiproliferative effects of HS can be worked out.

Table 2
Selection of regulated proteins with nuclear localisation

Protein name	Acc. #	M_r[a]	# of pep.	Ratio (H/L)	ICAT STDEV	Total ion score[b]	Best ion score[c]
RNA helicase A—human	gi|1082769	141983	2	0.57	0	67	48
EBNA-2 co-activator (100 kD)	gi|7657431	99628	2	0.78	0.021	91	55
Similar to heterogeneous nuclear ribonucleoprotein M	gi|14286202	44697	1	1.26	0	38	38
Heterogeneous nuclear ribonucleoprotein U	gi|14141163	90423	3	1.28	0.042	103	64
Heterogeneous nuclear ribonucleoprotein R	gi|12655185	71170	1	1.29	0	27	27
Programmed cell death 8 (apoptosis-inducing factor)	gi|14761510	48576	1	1.30	0	24	24
Splicing factor, arginine/serine-rich 7 (35 kD); 9G8	gi|5454054	27350	2	1.31	0.063	49	49
Small nuclear ribonucleoprotein polypeptide C	gi|4507127	17381	2	1.33	0.072	105	105
PML-1 protein	gi|190115	62008	1	1.34	0	[d]	[d]
Similar to myeloid-associated differentiation marker	gi|14758031	17049	2	1.36	0.14	71	42

[a] M_r: relative molecular weight.
[b] The total Mascot ionscore for all the identified peptides matching to the accession number.
[c] The highest Mascot ionscore matching to the accession number.
[d] See Fig. 5 for procedure for identification.

In summary, these results show that heparan sulfate with high content of (IdoA-2SO_4) and heavily sulfated glucosamine specifically inhibits MAP kinase activation with a subsequent change in the expression of nuclear proteins involved in cell cycle control.

Acknowledgments

This work was supported by grants from the Swedish Medical Research Council (11550), the Swedish Cancer Fund, the Swedish Society for Medical Research, the J.A. Persson, G. Nilsson, Greta and John Kock, A. Österlund and Anna-Greta Crafoord Foundations, Riksföreningen mot Rheumatism, Gustaf V.s 80 Årsfond, Heart-Lung Foundation and the Medical Faculty, University of Lund. The expert technical assistance of and Urzula Endresén is gratefully acknowledged. L. Pukac and M.J. Karnovsky were supported by N.I.H. grant HL17747. J.M. is supported by a Wennergren foundation postdoctoral fellowship and by financial support from the Lennanders stiftelse.

References

[1] M. Bernfield, M. Gotte, P.W. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, M. Zako, Annu. Rev. Biochem. 68 (1999) 729.
[2] M. Maccarana, B. Casu, U. Lindahl, J. Biol. Chem. 268 (1993) 23898.
[3] M.L. Fitzgerald, Z. Wang, P.W. Park, G. Murphy, M. Bernfield, J. Cell Biol. 148 (2000) 811.
[4] V. Kainulainen, H. Wang, C. Schick, M. Bernfield, J. Biol. Chem. 273 (1998) 11563.
[5] M. Lyon, G. Rushton, J.A. Askari, M.J. Humphries, J.T. Gallagher, J. Biol. Chem. 275 (2000) 4599.
[6] D.A. Pye, R.R. Vives, J.E. Turnbull, P. Hyde, J.T. Gallagher, J. Biol. Chem. 273 (1998) 22936.
[7] F. Safaiyan, U. Lindahl, M. Salmivirta, Eur. J. Biochem. 252 (1998) 576.
[8] E. Feyzi, F. Lustig, G. Fager, D. Spillmann, U. Lindahl, M. Salmivirta, J. Biol. Chem. 272 (1997) 5518.
[9] S.E. Stringer, J.T. Gallagher, J. Biol. Chem. 272 (1997) 20508.
[10] M. Yanagishita, V.C. Hascall, J. Biol. Chem. 267 (1992) 9451.
[11] H. Rahmoune, J.E. Turnbull, J.T. Gallagher, P.S. Rudland, D.G. Fernig, Biochem. Soc. Trans. 24 (1996) 355S.
[12] J. Malmstrom, G. Westergren-Thorsson, Glycobiology 8 (1998) 1149.
[13] G. Westergren-Thorsson, P.O. Onnervik, L.A. Fransson, A. Malmstrom, J. Cell Physiol. 147 (1991) 523.
[14] Y. Arroyo-Yanguas, F. Cheng, A. Isaksson, L.A. Fransson, A. Malmstrom, G. Westergren-Thorsson, J. Cell Biochem. 64 (1997) 595.
[15] D. Letourneur, B.L. Caleb, J.J. Castellot Jr., J. Cell Physiol. 165 (1995) 676.
[16] F. Redini, E. Moczar, E. Antoine, M.F. Poupon, Biochim. Biophys. Acta 991 (1989) 359.
[17] S.J. Busch, G.A. Martin, R.L. Barnhart, M. Mano, A.D. Cardin, R.L. Jackson, J. Cell Biol. 116 (1992) 31.
[18] F. Cheng, P. Petersson, Y. Arroyo-Yanguas, G. Westergren-Thorsson, J. Cell Biochem. 83 (2001) 597.
[19] L.A. Pukac, J.J. Castellot Jr., T.C. Wright Jr., B.L. Caleb, M.J. Karnovsky, Cell Regul. 1 (1990) 435.
[20] M.R. Stofega, C.L. Yu, J. Wu, R. Jove, Cell Growth Differ. 8 (1997) 113.
[21] T. Miralem, A. Wang, C.I. Whiteside, D.M. Templeton, J. Biol. Chem. 271 (1996) 17100.
[22] M.E. Ottlinger, L.A. Pukac, M.J. Karnovsky, J. Biol. Chem. 268 (1993) 19173.
[23] J.M. Herbert, M. Clowes, H.J. Lea, M. Pascal, A.W. Clowes, J. Biol. Chem. 271 (1996) 25928.
[24] L.A. Pukac, J.E. Carter, M.E. Ottlinger, M.J. Karnovsky, J. Cell Physiol. 172 (1997) 69.
[25] T.C. Wright Jr., L.A. Pukac, J.J. Castellot Jr., M.J. Karnovsky, R.A. Levine, H.Y. Kim-Park, J. Campisi, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 3199.
[26] L.A. Fransson, B. Havsmark, I.A. Nieduszynski, T.N. Huckerby, Biochim. Biophys. Acta 633 (1980) 95.
[27] L.A. Fransson, L.A. Nieduszynski, J.K. Sheehan, Biochim. Biophys. Acta 630 (1980) 287.
[28] G. Westergren-Thorsson, S. Persson, A. Isaksson, P.O. Onnervik, A. Malmstrom, L.A. Fransson, Exp. Cell Res. 206 (1993) 93.
[29] L.A. Fransson, A. Malmstrom, Eur. J. Biochem. 18 (1971) 422.
[30] J.R. Baker, L. Roden, A.C. Stoolmiller, J. Biol. Chem. 247 (1972) 3838.
[31] E. Jung, C. Hoogland, D. Chiappe, J.C. Sanchez, D.F. Hochstrasser, Electrophoresis 21 (2000) 3483.
[32] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, Nat. Biotechnol. 17 (1999) 994.
[33] G. Daum, U. Hedin, Y. Wang, T. Wang, A.W. Clowes, Circ. Res. 81 (1997) 17.
[34] U.L. Hedin, G. Daum, A.W. Clowes, J. Cell Physiol. 172 (1997) 109.
[35] N.S. Fedarko, M. Ishihara, H.E. Conrad, J. Cell Physiol. 139 (1989) 287.
[36] A.J. Sinclair, I. Palmero, G. Peters, P.J. Farrell, EMBO J. 13 (1994) 3321.
[37] E. Johannsen, E. Koh, G. Mosialos, X. Tong, E. Kieff, S.R. Grossman, J. Virol. 69 (1995) 253.
[38] P.D. Ling, D.R. Rawlins, S.D. Hayward, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 9237.
[39] K.L. Borden, Mol. Cell Biol. 22 (2002) 5259.
[40] A. Melnick, J.D. Licht, Blood 93 (1999) 3167.
[41] H.K. Lai, K.L. Borden, Oncogene 19 (2000) 1623.
[42] N. Cohen, M. Sharma, A. Kentsis, J.M. Perez, S. Strudwick, K.L. Borden, EMBO J. 20 (2001) 4547.
[43] X.F. Le, S. Vallian, Z.M. Mu, M.C. Hung, K.S. Chang, Oncogene 16 (1998) 1839.