The C-terminal Peptide of Chondroadherin Modulates Cellular Activity by Selectively Binding to Heparan Sulfate Chains

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The C-terminal peptide of chondroadherin modulates cellular activity by selectively binding to heparan sulfate Chains.

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Running title: Heparin binding proteins in the extracellular matrix*

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Keywords: chondroadherin, syndecan, cell spreading, cell binding, heparan sulfate, proteoglycan

Background:
Does the C-terminal cationic chondroadherin sequence bind cell surface proteoglycans to modulate cell behavior?
Result:
Chondroadherin and its C-terminal domain bind tightly to heparin and select proteoglycans eliciting signals and cell spreading.
Conclusion:
Peptides binding cell surface proteoglycans provide modulation of responses to cell receptor interactions.
Significance:
Provide insights into the role of extracellular matrix in regulating cellular activities.

SUMMAY

Chondroadherin, a leucine rich-repeat family member, contains a very C-terminal sequence CKFPTKRSKKAGRH[359], now shown to bind to heparin with a K_D of 13 µM. This observation led us to investigate if chondroadherin interacts via this C-terminal heparin-binding domain with glycosaminoglycan chains of proteoglycans at the cell surface. Cells was shown to bind this heparin binding peptide in FACS analysis and the interaction was to glycosaminoglycans as it was abolished when sulfation was inhibited by chlorate treatment of the cells. In separate experiments heparin and heparan sulfate inhibited the peptide interaction in a dose dependent manner. Using a human chondrosarcoma and a murine osteoblast cell line, heparan sulfate proteoglycans were identified as the cell surface receptors involved in the binding. Different binding syndecans were identified in the two different cell lines, indicating that the same protein core of a proteoglycan may have structural and functional differences in the attached heparan sulfate chains.

Upon binding to coated peptide cells spread, demonstrating engagement of the cytoskeleton, but no focal adhesion complex was formed. The number of cells adhering via their β_1 integrin receptor to collagen type II or chondroadherin was profoundly and rapidly enhanced by addition of the heparin binding peptide. The peptide added to the cells caused ERK phosphorylation showing triggered intracellular signaling.

The results show that heparan sulfate chain differ between various members of the proteoglycan families on a given cell, but also differ between the same proteoglycan on different cells with a potential for differential regulation of cellular activities.

Cell- matrix interactions play a vital role in tissue homeostasis. The chondrocyte is the only cell type in cartilage and occupies less than 10% of the total tissue volume. The cell is surrounded by an extensive extracellular matrix, which provides for key features of mechanical stability and resistance to load. A major constituent of this

matrix is the collagen network with type II representing about 95% of the collagens (1). The network overall organization and function also depends on associated collagenous (exemplified by collagen IX and XI) and non-collagenous proteins such as leucine rich repeat proteins e.g. fibromodulin, PRELP, asporin and decorin as well as other proteins including COMP (2-4) and matrilins (5). The major functions of the collagen network is to provide tensile strength and retention of the negatively charged aggrecan, the other major component of the tissue (6) Aggrecan is active in retaining water important for the cartilage resistance to deformation. A distinct collagenous network, with collagen VI as its major constituent, is located closer to the cells in the territorial matrix and interacts back to the collagen II based network as well as to aggrecan indirectly via a linker module of biglycan/decorin and a matrilin (7). Although the role of this network is not clear, its interactions indicate a function in tissue assembly and cell protection.

Matrix assembly and remodeling to adapt to new requirements is an important feature of cartilage, and essential in adapting to new load requirements and in correcting effects of wear and tear, e.g. fatigue. This process is orchestrated and finely tuned by the chondrocytes.

An important element in this regulation is the ability of the cells to use a diversity of surface receptors to interact with matrix proteins or protein fragments. These receptors include integrins (8), syndecans (9) collagens (10), such binding to hyaluronan (11,12), the discoidin family (11) as well as those for growth factors and cytokines (13). There are also molecules at the cell surface that when binding their ligand do not directly cause signals. Examples are hyaluronan and the GPI-anchored glypicans, which may still have roles in the communications of the cells with their surrounding. Several matrix proteins contain both integrin- and glycosaminoglycan- binding domains, e.g. fibronectin, and the formation of certain signaling complexes depends on targeting more than one cell surface receptor (14). There are a number of distinct integrins, where one of some 18 alpha chains combines with one of 8 beta chains to form the specific receptor. These have different ligands and elicit different responses when occupied by their particular interaction partner (8,15). In most cases, an interaction between a matrix protein and an integrin elicits tyrosine phosphorylation in a signaling cascade and interactions with the cytoskeleton. Downstream events are cell spreading, migration and/or division. Another class of signaling cell surface molecules are the syndecans. This family of four transmembrane heparan sulfate proteoglycans (16-18) commonly contains heparan sulfate chains, which can bind growth factors like bFGF and present them to their receptor. These glycosaminoglycan chains also bind a variety of matrix proteins including fibronectin, laminin, tenascin, vitronectin, collagens and thrombospondins 1 and 2 (19). Cells attach and spread on fibronectin with the formation of a complete focal adhesion complex requiring engagement of both integrin and syndecan receptors. This has particularly been studied for syndecan 4 in combination with integrin $\alpha_5\beta_1$ (14).

Chondroadherin belongs to the family of leucine rich repeat proteins. There are two forms of the protein in cartilage, only one containing the basic C-terminal extension peptide (20,21). Like other members of this family the protein binds to triple helical collagen with high affinity (22). Chondroadherin binds cells via the $\alpha_2\beta_1$ integrin. Upon binding, cells remain round which is unlike the spreading normally observed when matrix proteins bind to an integrin (23-25).

In this study we demonstrate that chondroadherin in solution binds to heparin structures including those of syndecans. Indeed, in the cells studied, binding appears selective for heparan sulfate among the glycosaminoglycans. The isolated chondroadherin C-terminal heparin binding domain (*hbd*) of 13 amino acids was shown to stimulate bound chondrocytes to spread and to prominently increase attachment to integrins with the formation of focal adhesion complexes.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Collagen type II was isolated from bovine nasal cartilage by pepsin digestion (Miller 4903-09). AlexaFluor 488 conjugated Streptavidin was from LifeTechnologies (Carlsbad, California, U.S).

Mouse monoclonal anti-vinculin antibodies (V9131) and Phalloidin-TRIC (P1951) were from Sigma. ALEXA-488 goat anti -mouse antibody, ALEXA-488 donkey anti-mouse antibody (A21202) and DAPI were from Molecular Probes (Eugene, OR, US). FITC goat anti-rabbit antibody (111-095-045) was from Jackson ImmunoResearch Laboratories, Inc. (West Glove,PA, US).

The chondroadherin specific rabbit antibody has been described (26) and the pan-syndecan antibody as well as the specific mouse syndecan 1-4 antibodies were kind gifts from Dr. Rapraeger, University of Wisconsin-Madison. Antibodies against human syndecan 1 (sc—12765) were from SantaCruz Biotechnology, human syndecan 2 (36-6200), human syndecan 3 (36-2400) and syndecan 4 (PAB9045) from Abnova, San Francisco, CA, US .

Anti rabbit HRP antibodies, phospho-ERK antibodies (phospho-p44/42MAP kinase) and total ERK1/ERK2 antibodies were from Cell Signaling Technology INC. Heparitinase, (Heparitinase I) and chondroitinase ABC were from Seikagaku (Japan). COMPLETE®, EDTA-free Protease Inhibitor Cocktail Tablets were from Roche Diagnostic GmbH (Germany).

Heparin was from Sigma, US. Glycosaminoglycans were those obtained from the NIH-standard.

Expression and purification of recombinant protein—Recombinant chondroadherin was expressed in (U293) EBNA cells (22) or in Escherichia coli M15 (pREP4) and purified as described (24).

Recombinant chondroadherin expressed without the cationic most C-terminal part of 13 amino acids with the amino acids PGWAA as a C-terminal extension was generated using the primer 5'- ATGGTCCGCCCAATGCTC
-3' with a flanking *HindIII* site and 5'-ACGCCTTCCGCAGCTGC
CCGGGCTGGGCTGCCTAG-3' with a flanking *BamH I* site and expressed in EBNA cells in the same manner as described in (22).

Mass spectrometry—Mass spectrometry (MS) was performed using a Bruker Scout 384 Reflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS instrument. The mass spectrometer was used in the positive ion mode with delayed extraction and an acceleration voltage of 26 kV. Peptide samples were analyzed using the reflector detector and 50-150 single-shot spectra were accumulated for improved signal to noise ratio. The ProFound software was used to identify the obtained peptides.

Synthetic peptides—Peptides corresponding to the C-terminal heparin-binding domain (*hbd*) CKFPTKRSKKAGRH^{359} and biotin-CKFPTKRSKKAGRH^{359} were synthesized and purified by reversed phase chromatography by Schafer-N (Copenhagen, Denmark). Biotin-CRFPTRKSRRAGKH^{359} was synthesized and purified by GenScript (US). The structures of the peptides were verified by mass spectrometry.

Heparin binding—Intact recombinant Chondroadherin (30 μg, expressed in EBNA-cells) diluted in PBS (150 mM NaCl, 5 mM sodium phosphate, pH 7.4) and the chondroadherin protein without the basic most C-terminal amino acids (t-CHAD) (200 μg) as well as the *hbd*-CKFPTKRSKKAGRH^{359} (30 μg), were passed over a 1 ml Heparin-Sepharose Column (Amersham Pharmacia), which was subsequently washed with 3 volumes of starting buffer, 5 mM sodium phosphate, 0.075 M NaCl, pH 7.4 (0.15M NaCl in 5 mM sodium phosphate, pH 7.4 in the case of t-CHAD) and eluted with a gradient of 20 ml of 0.075 -1 M NaCl (0.15M-1M in the case of t-CHAD) in 5 mM sodium phosphate, pH 7.4.

Isothermal titration calorimetry (ITC)—ITC was performed using a MicroCal VP-ITC microcalorimeter (MicroCal, Northampton, MA).

Heparin (H0777, Sigma, USA) was dissolved in PBS at 0.3 mM (4.5 mg/ml) calculated on an average mass of 15,000 Dalton. The short *hbd*-KFPTKRSKKAGRH^{359} was dissolved at 0.1 mM in the same buffer and filled into the calorimeter cell. The heparin solution was titrated into the peptide solution by injections of 5 μl with a delay of 180 seconds between the injections. The effect of dilution of the heparin solution in the titration cell was controlled by a blank titration with heparin into the buffer solution. All experiments were performed at 30 ºC. In order to abolish noise in the measurements of the sample, the heparin blank titration was subtracted before the calculation. The binding constant was evaluated by using the ITC data analysis in Origin® software supplied with the Microcal VP-ITC instrument.

Cells and cell culture—The cell line (105kc), originating from a human chondrosarcoma, was a kind gift from Dr. Sven Inerot, The Sahlgrenska University Hospital, Gothenburg, Sweden. The 105kc cells were cultured in a mix of 40% Dulbecco's Modified Eagle Medium, 40% Minimum Essential Media (MEM) alpha and 10% Ham's F12, supplemented with 100 nM hydrocortisone, 100 ng/ml insulin, 10% fetal bovine serum, 25 μg/ml ascorbic acid, 50 IU of penicillin and 50 μg/ml streptomycin (Life Technologies, Inc.).

Mouse osteoblasts (MC3T3-E1) were cultured in Ham's F12, 10% fetal bovine serum.

Human chondrocytes (kindly provided by Dr. Recklies, Shriners hospital, Montreal, Canada)

were prepared from cartilage obtained at autopsy from a 2-year-old individual. Cells were isolated as described previously (27) and stored frozen after passage 1. For the work described here frozen chondrocytes were expanded and used at passage level 5. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 μg/ml penicillin plus 100 units/ml streptomycin.

Bovine articular chondrocytes were isolated by collagenase (CLS1; Worthington Biochemical Corp., Lakewood, NJ, US) digestion of articular cartilage from 4-6 month old calves as described (28). Briefly, cartilage slices were digested for 16 hours with the crude collagenase in EBSS (Earle´s balanced salt solution, GIBCO BRL, Gaitersburg, MD, US). The cells were filtered through a 100 μm nylon filter, and washed three times in PBS containing 0.2% BSA (11930, SERVA electrophoresis, Germany).

To harvest cells, culture dishes were rinsed three times with Ca^{2+}/Mg^{2+}-free PBS and briefly incubated with 0,5% trypsin and 1 mM EDTA. Detached cells were suspended in PBS containing 1 mg/ml trypsin inhibitor (Sigma, US) and then washed in PBS. Cells contain membrane bound collagens, e.g. type XIII (29), which were removed by treatment with collagenase (CLSPA, Worthington Biochemical Corp., Lancewood, NJ) at 100 U/ml in PBS for 30 min at 37ºC. Cells were then washed in PBS.

Sodium Chlorate treatment of the human 105kc cells—Human 105kc cells were plated in culture dishes and allowed to adhere overnight. The cells were rinsed with PBS followed by Ham´s F12 medium. The cells were incubated for 24 hours in Ham´s F12 medium containing either 0.13 M NaCl or 0.13 M Sodium chlorate ($NaClO_3$). The cells were detached by a brief incubation with 5 mM sodium phosphate pH 7.4, 0.5 mM EDTA, 0.3 M NaCl and directly used in FACS analysis assay of peptide binding as described below. In parallel, cells were incubated in the same manner as above but in presence of ^{35}S-sulfate. Cell extracts were separated by SDS-PAGE and analyzed for presence of incorporated sulfate by autoradiography.

Immunochemical staining—Chamber slides (4 chamber; Lab-Tek®, Nunc Inc., Naperville, IL) were coated overnight with 5 μg/ml chondroadherin in 4 M Guanidine-HCl, 50 mM sodium acetate, pH 5.8 or 33 μg/ml (20 μM) *hbd*-CKFPTKRSKKAGRH^{359} and blocked for nonspecific binding with 0.5% BSA in PBS for 3 hour. Human chondrosarcoma cells (105kc, 100 000 cells/chamber) were allowed to adhere and spread for 1 hour with or without the peptide *hbd*-CKFPTKRSKKAGRH^{359}. Unbound cells were removed and adherent cells were fixed in 2% paraformaldehyde in PBS. Cells were permeabilized with 0.5% triton X-100 in PBS and background binding was blocked with 0.1% BSA in PBS followed by incubation with an anti-vinculin antibody (1:200 in PBS, 0.1% BSA) for 1 hour. After washing in PBS, sections were incubated with ALEXA-488 conjugated antibodies (1:200 in PBS, 0.1% BSA) and Phalloidin-TRIC (50 μg/ml) for 30 minutes. Washed sections were then incubated with DAPI (1:2000 in PBS), washed twice with PBS and once in Milli-Q-water and mounted with AquaPerm Mounting Medium (IMMUNON^{TM}, Thermo Fisher Scientifics, US). All incubations were carried out at room temperature. Microscopy was carried out using a Leica DM 4000B microscope equipped with a DFC420 digital camera.

Cell adhesion—Tissue culture 48-well or 5 cm dishes (Nunclon, Nunc, Denmark) were coated overnight with 5 μg/ml or 0.04 μg/ml full-length chondroadherin in 4 M Guanidine-HCl, 50 mM sodium acetate, pH 5.8. Alternatively, coating of the *hbd*-CKFPTKRSKKAGRH^{359} was at 0.16, 0.6, 2.5, 10 or 20 μM in PBS. The C-terminally truncated Chondroadherin (t-CHAD) was coated over night at 5 μg/ml in PBS. Collagen type II was diluted with PBS from a 0.5 M acetic acid stock solution immediately prior to coating at 5 μg/ml or 0.04 μg/ml. Coated surfaces were blocked for non specific binding with 0.5% BSA in PBS for 3 hours.

To test cell binding, the chondrosarcoma cells or bovine chondrocytes were suspended in PBS containing 0.1% BSA, 25 U/ml collagenase (CLSPA-grade, Worthington) and added to the wells (50 000 cells/well), in the presence (250 μg/ml, 150 μM) or absence of the synthetic peptide. Non-adherent cells were removed after 1 hour and bound cells were quantified by measuring lysosomal N-acetylglucosaminidase (30). Cell adhesion and spreading were visualized by light microscopy.

Flow cytometric analysis—Human chondrosarcoma cells (105kc) were detached with 0.5 mM EDTA in 5 mM sodium phosphate pH 7.4, 0.3 M NaCl prior to incubation for 30 min on ice with the synthetic *hbd*-peptide biotin-CKFPTKRSKKAGRH^{359} or variant peptide biotin- CRFPTRKSRRAGKH^{359} in complex with streptavidin-Alexa Fluor 488. For the inhibition

assay, NIH reference standard, glycosaminoglycans (Heparan sulfate, Heparin, CS-A, CS-B, CS-C or keratan sulfate, at 100, 20, 4, 0.8 and 0.16 µg/ml) were pre-incubated for 30 min on ice with the peptide-streptavidin Alexa Fluor 488 complex. The glycosaminoglycan-peptide mixture was then allowed to interact with the cells for 30 min. Alternatively, antibodies recognizing the different syndecans were allowed to interact with the cells followed by either FITC conjugated goat anti rabbit antibody or Alexa Fluor 488 conjugated sheep anti mouse antibody. All cells were washed in PBS, 0.5% BSA after incubations. Background staining was assessed by omitting the peptide or the primary antibody. Antibody binding to cells was analyzed using a FACS Calibur (BD Bioscience).

Cell signaling—Tissue culture 6-well dishes (Costar, Corning Life Science, Netherlands) were coated overnight with 5 µg/ml chondroadherin in 4 M Guanidine-HCl, 50 mM sodium acetate, pH 5.8, or 20 µM of the *hbd*-CKFPTKRSKKAGRH^{359} in PBS. The dishes were blocked for non-specific binding with 0.5% BSA in PBS for 1 hour. To determine ERK phosphorylation, the human chondrocytes were serum starved for 48 hour prior to the experiment. To harvest cells, culture dishes were rinsed three times with Ca^{2+}/Mg^{2+}-free PBS and the cells were incubated for 30 seconds with 0.5% trypsin, 1 mM EDTA. Detached cells were suspended in PBS containing 1mg/ml trypsin inhibitor (Sigma), then washed in PBS, suspended in PBS containing 0.1% BSA, added to the wells coated with chondroadherin or BSA (500 000 cells/well, 2 ml/well) and allowed to adhere for 1 hour at 37°C. All cells (bound and unbound) were collected and lyzed with SDS-PAGE (150 µl) sample buffer. Aliquots (30 µl) of the cell lysates were separated by linear 10% SDS-PAGE under reducing conditions and transferred electrophoretically to nitrocellulose membranes. Blots were incubated with the phosphorylation-specific ERK antibody at dilutions recommended by the manufacturer. Following visualization using the ECL chemiluminescence system (GE Healthcare, Uppsala, Sweden) antibodies were removed from the membranes by incubation for 1 hour at room temperature in a 0.2 M Tris/glycine buffer, pH 2.8 (containing 0.1% SDS and 0.1% Tween 20). The membranes were re-probed with an antibody to determine total ERK1/ERK2. Gel-Pro Analyzer® Software (Media Cybernetics, US) was used for scanning of films representing the exposure of the blots for quantification of phosphorylation.

Coupling of affinity matrix—Recombinant chondroadherin (expressed in EBNA-cells) was coupled to Mini-Leak agarose (Kem-En-Tec, A/S, Denmark) (2.5 mg/ml of agarose) according to the manufacturer's instructions. The control agarose was treated in the same manner but with no protein added. The synthetic *hbd*-CKFPTKRSKKAGRH^{359} was coupled to UltraLink agarose (Pierce, Thermo Fisher Scientifics, US) via the N-terminal cysteine residue and excessive binding sites were blocked according to the instructions of the manufacturer. Control matrix was treated in the same manner omitting the peptide.

Surface labeling—Human chondrosarcoma cells (105kc, 20x10^6/ml), suspended in PBS were incubated with EZ-link sulfo-NHS-LC biotin (Pierce, Thermo Fisher Scientifics, US) at a final concentration of 1 mg/ml, for 1 hour at room temperature. The cells were then incubated for 10 minutes in 0.1 M glycine in PBS to block further reactivity of free biotin, washed three times in PBS and the membrane fraction was isolated as described below (membrane preparation).

Membrane preparation for proteoglycan isolation—Unlabeled or biotin labeled 105kc cells were suspended and homogenized in 10 mM KCl, 1 mM EDTA, 20 mM Tris/HCl, pH 7.4 (1ml/10^6 cells). Nuclei were removed by centrifugation at 1,500 g for 5 minutes. NaCl (0.15 M) was added to the supernatant and the membrane fraction was collected by centrifugation at 50,000 g for 30 minutes. The pellet was suspended in 8 M urea, 2% Triton-X-100 in 25 mM Tris pH 7.4, 0.15 M NaCl (TBS) supplemented with COMPLETE® proteinase inhibitor. The lysate was cleared by centrifugation at 14,000 g for 20 min.

The MC3T3-E1 cells were prepared in the same manner as above except that 1 M NaCl was added to the supernatant after the first centrifugation. The pellet was in this case suspended in 6 M urea, 1% Triton-X-100, 3% Tween 20, 3% NP-40, 0.5% sodium deoxycholate in 25 mM Tris pH 7.4, 0.15 M NaCl (TBS) supplemented with COMPLETE® for 1 hour in 4 °C and cleared as above.

Affinity purification of chondroadherin binding proteoglycans—The affinity matrices (1 ml) of control- and chondroadherin were packed in separate mini columns (Bio-Rad, Hercules, CA) and equilibrated with 20 volumes running buffer (6 M urea, 1% Triton-X-100 in TBS, pH 7.4),

supplemented with COMPLETE®. Lysates of biotin labeled cells in the same buffer were passed twice over the control-column and then incubated overnight with the affinity matrix column with continuous end over end mixing at 4°C. The affinity and control matrix columns were washed with 20 volumes of running buffer and eluted (1 ml fractions) with 0.5 M NaCl, 6 M urea in TBS, pH 7.4, supplemented with COMPLETE®. Eluted proteins were precipitated with ethanol and re-suspended in heparitinase buffer (50 mM Hepes, 50 mM sodium acetate, 150 mM NaCl, pH 6.5). Half of the material was digested with heparitinase (0.3 mU) and chondroitinase ABC (100 mU) at 37°C overnight. Undigested and digested materials were separated by a 4-12% gradient SDS-polyacrylamide gel under reducing conditions and electro-transferred to a PVDF-membrane (Pall Corporation, US). Non-specific binding to the membrane was blocked with 2% BSA in 10 mM Tris-HCl, 0.15 M NaCl, 2% Tween, pH 7.4 (blocking buffer). Biotin labeled proteins was visualized after incubation with streptavidin-HRP (1:5000 in blocking buffer) by chemiluminescence detection using the ECL system.

Identification of syndecans on the human chondrosarcoma 105kc cells and MC3T3-E1 cells—To demonstrate that 105kc and MC3T3-E1 cells express syndecan molecules they were homogenized in PBS - 1% Triton and COMPLETE® proteinase inhibitor. The lysates were cleared by centrifugation (12000 g, 20 min) and precipitated with 3 volumes of methanol.

The precipitates were dissolved in heparitinase buffer and digested at 37°C as described above. Syndecans present and expressed by the 105kc or MC3T3-E1 cells were separated on a 10% SDS-polyacrylamide gel under reducing conditions and electrophoretically transferred to a PVDF membrane. The membrane was blocked for non-specific binding with blocking buffer. Syndecans present were detected with the various syndecan antibodies (1 μg/ml) followed by the appropriate secondary HRP-tagged antibody (anti rabbit or anti mouse antibody) and detected by chemiluminescence using the ECL system.

Affinity purification of membrane proteoglycans binding to the synthetic hbd-CKFPTKRSKKAGRH^{359}—The procedure for affinity purification was the same as that used for the biotin tagged proteoglycan, except that the 105kc cells were unlabeled and the peptide was used for affinity. The cell membrane fraction of the MC3T3-E1 cells was applied to the peptide column equilibrated in 6 M urea, 1% Triton-X-100, 3% Tween 20, 3% NP-40, 0.05% sodium deoxycholate in 25 mM Tris pH 7.4, 0.15 M NaCl (TBS) supplemented with COMPLETE® and washed with the same buffer. Proteins present were eluted with 1.5 M NaCl in the same buffer as above and precipitated with ethanol, re-suspended in heparitinase buffer and divided into two aliquots where one was digested with heparitinase at 37°C overnight. Digested materials were separated by linear 10% SDS-polyacrylamide gels (reducing conditions) and electro-transferred to a PVDF-membrane. The western blot procedure for detecting the presence of syndecans was performed as described above.

Immunoprecipitation—Biotin labeled cell surface material was affinity purified using immobilized chondroadherin as described above. To change buffer, fractions were passed over a PD-10 column (Amersham-Pharmacia Biotechnology) equilibrated and eluted with 0.1% triton X-100 in TBS supplemented with COMPLETE®. Eluted material was incubated overnight with a pan-syndecan antibody (5 μg/ml) and the immune complex was collected using protein-A-Sepharose. The gel beads were washed 3 times with heparitinase buffer, re-suspended in the same buffer and digested with heparitinase at 37°C for 16 hours with two additions of 0.3 mU each, two hours apart. Immunoprecipitated proteins were eluted with SDS sample buffer and separated by SDS-PAGE on 10% gels (reducing conditions), and electro-transferred to PVDF-membranes. The membranes were blocked with blocking buffer and proteins were visualized after incubation with streptavidin-HRP (1:5000 in blocking buffer) by chemiluminescence detection using the ECL system.

RESULTS

Chondroadherin binding to heparin—The very C-terminal part of chondroadherin (KFPTKRSKKAGRH) contains a heparin binding consensus sequence similar to the one described by Cardin *et al* XBBXBX (31) and the corresponding synthesized peptide interacts with heparin (21). It is however not known if the sequence is exposed in the intact protein. To address this, purified recombinant chondroadherin was passed over a Heparin-Sepharose column followed by a wash with 5 mM sodium phosphate, 0.075 M NaCl, pH 7.4 and then eluted with a gradient from 0.075 to 1 M NaCl. We found that chondroadherin (pI 9.43) indeed binds

to heparin and elutes as a broad peak with maximum at 0.6 M NaCl (fig. 1).

For comparison chondroadherin, lacking the C-terminal heparin-binding domain (pI 9.02), was expressed in EBNA cells in the same manner as the full-length chondroadherin protein. The truncated protein is still very basic and binds to heparin but eluted from the column at a lower ionic strength of 0.5 M NaCl (Supplemental data, fig. 1). This demonstrates that the C-terminal domain contributes to heparin binding of this overall very basic protein.

Further prof was obtained in studies of the isolated peptide in the form of synthetic *hbd*-CKFPTKRSKKAGRH^{359}. This peptide was found to elute at 0.8 M NaCl from the Heparin-Sepharose column (fig. 1, inserted), confirmed by MALDI-TOF MS.

ITC measurement of binding of the *hbd*-CKFPTKRSKKAGRH^{359} peptide to heparin was performed to evaluate the interaction. This identified a K_D of 13 μM, which indicates a high affinity between the two molecules (supplemental data, fig. 2).

Cellular interaction of the heparin binding peptide analyzed by FACS—Heparin binding domains in e.g. fibronectin have the potential to interact with cell surface proteoglycans. FACS analysis was performed to evaluate if the heparin-binding domain of chondroadherin had similar features. The *hbd* biotin-CKFPTKRSKKAGRH^{359} was incubated with 105 KC cells and binding was evaluated by flow cytometry. The peptide showed strong binding already at the lowest dose tested (5 μM) (fig. 2A). To verify that the interaction was mediated via sulfated glycosaminoglycans of cell surface proteoglycans, cells were treated with sodium chlorate. The cells were shown to not incorporate any sulfate to the cell surface proteoglycans (fig. 2B, insert). The *hbd* biotin-CKFPTKRSKKAGRH^{359} peptide showed little remaining affinity to cells indicating that binding requires sulfated glycosaminoglycans at the cell surface (fig 2B).

To further study the specificity of the binding of the *hbd*-CKFPTKRSKKAGRH^{359} peptide, a mutated variant was designed were all arginine residues were exchanged for lysine residues and all lysine residues exchanged for arginine residues (CRFPTRKSRRAGKH). The peptides were compared for binding to the human chondrosarcoma cells by FACS analysis. Interestingly, the original peptide showed four times higher affinity in binding to the cell surface proteoglycans (fig. 3A and B).

Identification of membrane proteoglycans binding to Chondroadherin and the synthetic hbd-CKFPTKRSKKAGRH^{359}—As the heparin-binding domain was found to be exposed in the intact protein in solution and the *hbd* biotin-CKFPTKRSKKAGRH^{359} interacts with cell surface components, we set out to identify the receptor responsible for this interaction. Human chondrosarcoma cells (105kc) were surface labeled with biotin, membrane proteins were isolated and affinity purified on chondroadherin-agarose. Affinity purification was carried out at high urea concentrations in order to abolish protein-to-protein interactions thereby focusing on glycosaminoglycan to protein interactions. Bound material was eluted with 0.5 M NaCl, separated on a 4-12% gradient SDS-polyacrylamide gel and detected by Western blot analysis before and after a combined heparitinase and chondroitinase ABC treatment (fig. 4). Untreated affinity purified material migrated as a diffuse band with a molecular weight range distributed around 250 kDa. The size was reduced to a distinct band corresponding to approximately 50 kDa after enzyme digestions, clearly demonstrating that the component represents a proteoglycan (fig. 4). In a separate experiment similar results showing one major band at 50 kDa were obtained after digestion with heparitinase only (data not shown), demonstrating that the proteoglycan carries heparan sulfate chains. The data suggests that syndecans at the cell surface represent the receptor. Indeed the 105kc cells appear to express most, if not all of the syndecans (fig. 5 and 6A). The presence of the four proteoglycans was demonstrated by FACS analysis (fig. 5) using specific antibodies. In parallel, their core proteins were identified using a pan-syndecan antibody in western blots of cell extracts after heparitinase digestion (fig. 6A). This experiment further verified that these syndecans carry heparan sulfate side chains by the necessity to apply digestion with heparitinase to visualize their core proteins as distinct bands.

To identify the ligand, affinity purification was repeated as above by using the chondroadherin column and the eluted material immune precipitated using the pan-syndecan-antibody. The antibody primarily precipitated a protein of about 50 kDa after heparitinase digestion, whereas no distinct protein was detected corresponding to this size from the control-column (fig. 6B).

In further support of the binding to the C-terminal peptide, a heparitinase digest of material affinity purified on the immobilized *hbd*-CKFPTKRSKKAGRH^{359} peptide likewise revealed a prominent component of about 50 kDa, (fig. 6C), while no material was found in the eluate from the control column. Proteins eluted from the affinity columns were separated by SDS-PAGE followed by western blotting. An attempt to identify the proteoglycan receptors was performed using specific antibodies to the four human syndecans. None of the specific antibodies showed reaction with the material eluted from the columns. The antibodies also failed to recognize the syndecans in total extract from the cells even though the pan-syndecan antibody clearly identified proteins corresponding to the size of the four syndecan receptors. Only the antibody to mouse syndecan 4 was found to react with a protein of the expected size in western blotting (fig. 6A).

In a further attempt to elucidate the specificity, mouse osteoblast cells MC3T3-E1 were used in affinity purification over the *hbd*-CKFPTKRSKKAGRH^{359} immobilized via its cysteine. Mouse specific antibodies directed to syndecan 1 and 4 identified both receptors in total extracts from the cells (fig. 7A). Interestingly, only syndecan 4 was detected in affinity purified proteoglycans from the cells indicating selective binding of one of the cell surface proteoglycans (fig. 7B). The syndecan 4 receptor identified on the MC3T3-E1 cells is of a different size compared to the receptor isolated from human 105 KC cells possibly representing a differently spliced receptor on these cells. The data further support the results from the chlorate treatment to demonstrate that the peptide interacts with glycosaminoglycan chains with a specific sulfation pattern rather than with specificity for the core protein carrying the particular glycosaminoglycan.

Signaling elicited by binding of the heparin ligand peptide—To determine whether the interaction elicits intracellular signals, intact chondroadherin and the isolated heparin binding domain CKFPTKRSKKAGRH^{359} were coated separately onto cell culture plates. Surface coated with albumin only was used as a control. All cells, both bound and un-bound, were collected, lyzed and analyzed by western blot using antibodies specific for P-ERK and total ERK (fig. 8A, 9A) respectively. A representative example of the gels scanned is shown in fig. 8 C. Indeed the binding via the cell surface syndecan to the chondroadherin heparin-binding domain increased ERK phosphorylation 5-fold compared to the control (fig. 8A). One consequence of the binding was a novel observation of a dose dependent cell spreading on a heparin sulfate ligand (fig. 8B).

We have previously demonstrated that cell adhesion to intact chondroadherin or its integrin binding sequence does not induce spreading. This led us to investigate if the heparin-binding domain identified is masked when the protein is passively coated to cell culture plastic. In support, chondroadherin coated onto cell culture plates as described previously failed to bind biotin tagged heparin, indicating that the heparin binding domain indeed is masked when chondroadherin binds to the surface (data not shown). Adhesion to chondroadherin induced tyrosine phosphorylation of ERK at a level some 4 times over that of the control (fig. 9A). As expected this binding did not induce any cell spreading (fig. 9B, top panel). Interestingly, when the heparin binding peptide was added in solution to cells attaching via the $\alpha_2\beta_1$ binding domain of chondroadherin spreading was induced and formation of focal adhesion complex was demonstrated by the typical organization of vinculin and actin (fig. 9B, bottom panel). For comparison cells demonstrate spreading when binding to this heparin binding peptide, but no formation of focal adhesion complex (fig. 9B, middle panel) indicating that engagement of both integrin and syndecan receptors is required for the formation of focal adhesion complexes. The addition of the heparin binding peptide resulted in a further increased ERK-phosphorylation, compared to the chondroadherin integrin binding alone (fig. 9A).

Cell adhesion to chondroadherin is enhanced by the hbd-CKFPTKRSKKAGRH^{359}—Wells were coated with either chondroadherin or collagen type II at 0.04 and 5 μg/ml and remaining sites were blocked with BSA. 105kc cells were allowed to adhere in the presence of the *hbd*-CKFPTKRSKKAGRH^{359} to determine whether binding and spreading were modulated. Adhesion and spreading to collagen type II was pronounced at high coating concentrations (5 μg/ml) and was marginally increased by the peptide (fig. 10B). However, using lower coating concentrations of collagen type II, we found that both the number of cells adhering and their degree of spreading markedly increased in the presence of the peptide (fig. 10B, histograms). Adhesion to chondroadherin at any of the coating concentrations increased the number of cells

adhering in the presence of the peptide (fig. 10A). Cells plated on chondroadherin lacking the C-terminal heparin binding domain showed the same behavior as when plated on full length chondroadherin both with and without the heparin binding peptide present (Supplemental data, fig. 3), confirming that the sequence is not exposed upon binding to plastic. Bovine primary chondrocytes were used to confirm that the peptide likewise increases binding of primary cells. Indeed adhesion similarly increased when cells were pre-treated with the heparin binding peptide CKFPTKRSKKAGRH^{359} (fig. 11).

The effect of glycosaminoglycans on cell interaction of the hbd-CKFPTKRSKKAGRH^{359}— The influence of glycosaminoglycans on the binding of the synthetic *hbd*-CKFPTKRSKKAGRH^{359} was studied to define the degree of specificity. The synthetic *hbd*-CKFPTKRSKKAGRH^{359} labeled with streptavidin Alexa Fluor 488 was incubated with either heparin, heparan sulfate, CS-A, CS-B, CS-C or keratan sulfate at different concentrations followed by analysis of the interactions with the 105 kc cells by FACS. Binding of the peptide to the cells decreased in a dose dependent manner in the presence of heparin and heparan sulfate (fig. 12A and B) indicating a binding to the peptide via heparan sulfates at the cell surface. Very high concentrations of CS-B (dermatan sulfate) did result in a similar degree of inhibition of peptide binding to cells while CS variants and keratan sulfate showed no inhibition. This inhibition by dermatan sulfate may be due to the fact that this glycosaminoglycan contains stretches of highly sulfated domains in analogy with the highly sulfated domains in heparan sulfate.

DISCUSSION

Cell adhesion to chondroadherin has been shown to be mediated via the integrin $\alpha_2\beta_1$ (23, 24). Upon binding the cells do not spread unlike effects observed when they adhere to other matrix proteins like collagen or fibronectin. One difference is that many, if not most, of the integrin binding proteins also contain an additional domain with capacity to bind heparan sulfate present on the cell surface as a part of e.g. syndecans. It has been shown that cells adhering to the isolated integrin binding domain of fibronectin fails to form focal contacts, but addition of the isolated heparin binding domain of the protein, induced spreading and focal contact formation (32).

Earlier work has shown that spreading of cells bound to chondroadherin can be induced upon phorbol 12-myristate 13-acetate (PMA) stimulation (23). A synthetic peptide of the very C-terminal part of chondroadherin contains a cluster of basic amino acids having heparin-binding properties (21). This led us to investigate if the chondroadherin protein has the potential to bind to heparin and if this domain may elicit cell spreading.

In initial experiments we found that the bacterially expressed chondroadherin when passively bound to a plastic surface appeared not to present the heparin binding structure, since biotinylated heparin showed no binding (data not shown). In another set of experiments, chondroadherin was passively coated onto a polystyrene surface of an Attana® quartz microbalance and shown not to bind heparin, while the coated isolated peptide representing the heparin-binding domain showed binding to the glycosaminoglycan. At the same time in isothermal titration calorimetry experiment the equilibrium constant in the interaction between heparin and the short *hbd*-KFPTKRSKKAGRH^{359} peptide was measured and had a high K_D of 13 μM, and we could also show that chondroadherin in solution can bind to heparin. The mammalian expressed protein eluted in a broad peak from the heparin column, while the peptide was sharp. The difference could result from the presence of forms with the heparin-binding domain cleaved off. When, in analogy with other SLRPs (33), a proportion of the chondroadherin molecules dimerizes, complexes with different contents of the heparin-binding domain are formed with most likely slightly different chromatographic properties, resulting in the broad peak from the heparin column.

The very C-terminal CKFPTKRSKKAGRH^{359} peptide sequence interacts with heparin and mediates cell binding that distinctly depends on sulfated proteoglycans at the cell surface, since preventing sulfation abolish binding. This peptide, as well as the intact chondroadherin molecule, was used to selectively purify cell surface ligands by affinity chromatography under conditions of high urea concentration to prevent non-ionic interactions. We demonstrated using a pan-syndecan antibody that primarily one of the syndecans present on human 105kc cells bound to the heparin-binding domain. Judging by the size, syndecan 3 but not 4 is a possible candidate. In further experiments using a battery of commercial syndecan antibodies we could not identify which

of the 4 syndecan that was involved in binding to the peptide because of lack of specificity in western blots of the available antibodies to the human syndecans. In another set of experiment with MC3T3-E1 cells using antibodies to mouse syndecans we could also show selective binding to syndecans to the chondroadherin heparin binding peptide. These cells were shown to present syndecan 1 and 4 while only syndecan 4 was bound and affinity purified. It is of particular interest that of two or more only one of the syndecans on the cell appears to bind to the chondroadherin sequence. In further support, FACS analysis with specific antibodies verified the presence of all 4 syndecans at the cell surface on the human chondrosarcoma cells (fig. 5). Together the data provide strong evidence for a preferred binding of the peptide to the glycosaminoglycan chains of only one of the syndecans on these cells. This indicates that the heparan sulfate (or GAG chains) chains of the four syndecans on a cell are not necessarily identical. We could in a separate set of experiments show that the binding was specific to heparan sulfate since only heparin and heparan sulfate efficiently reduced binding of the peptide to the cell surface proteoglycan (fig. 12). Since binding is mediated via the heparan sulfate chains it appears that their structure differs between the various syndecans on the cell lines studied (34). The results provide a focus on aspects governing synthesis of these glycosaminoglycan side chains and how the structure of these oligomers of repeat disaccharides differs. An important factor is sulfation, where alterations could be coupled to different levels of epimerization of the uronic acid. Mechanisms to form different heparan sulfate chains may involve timing of the production of the particular syndecan or possibly different compartmentalization of the synthetic process. The observations point at a mechanism whereby in a unique way subtle specificities for cellular interactions can be created. This offers specific cellular options for sensing events in their surrounding matrix.

Integrins are the most extensively characterized adhesion receptor family. The syndecans are now also recognized as important cell surface adhesion co-receptors that can actively participate in adhesion and signaling (18,35,36). The *hbd*-CKFPTKRSKKAGRH^{359} was shown to profoundly stimulate adhesion, spreading and migration upon integrin mediated to binding chondroadherin as well as on collagen *in vitro*. Similar effects on cell adhesion have been shown after (PMA) treatment (14,23,37,38). PMA is known to activate protein kinase C signaling and thereby stimulate adhesion and spreading in a process involving syndecan 4 and integrins (14). We now show that adhesion to the *hbd*-CKFPTKRSKKAGRH^{359} alone will promote cells to adhere firmly and interestingly also spread. In this case with the 105kc, one of the syndecan proteoglycans involved is different from syndecan 4 that has previously been implicated in rearrangement of the cytoskeleton. This active peptide sequence in chondroadherin is rather short compared to other heparan sulfate binding domains investigated. However, in a study of smaller segments of the large heparin-binding domain of fibronectin it was shown that a short peptide was sufficient to generate the effects (14). It is clear that cell binding via syndecan to the heparin binding domain of chondroadherin leads to potent activation of intracellular signaling documented as tyrosine phosphorylation of ERK. We could further show that binding also induced cell spreading (fig. 8B and 9B) but for formation of the focal adhesion complex additional signaling events via integrin binding was required, while binding to the integrin alone neither induced cell spreading nor formation of the focal adhesion complex (fig. 9B and 10).
The combined action of these signaling events present interesting possibilities for fine tuning of cellular responses in relation to the microenvironment and interactions at the cell surface.

The nature of the signals elicited by the binding of the peptide to the heparan sulfate chains of syndecan is not clear. The very rapidly induced increased cell binding and cell spreading by the peptide indicate that protein synthesis is not involved. The short peptide (corresponding to an extended length of some 40 Å) will bind to a small segment of the heparan sulfate chain only (corresponding to 4-5 disaccharides). We consider it unlikely that a conformational change is mediated via the flexible glycosaminoglycan chain to the core protein and then to the cytosolic side of the proteoglycan. It is possible that the peptide will influence interactions and network formation at the cell surface, which should represent a very rapid process, similar to the effects we observe. Indeed interactions between the syndecan core protein and integrin mediated by synstatin has been shown to modulate cells behavior (39). In previous work it has been suggested that the much larger fibronectin derived heparan sulfate binding domain elicits clustering

of the syndecan. Ensuing interactions with the core proteins have been proposed to lead to the conformational changes. We now find that this very short peptide binds tightly to heparin and promotes all the cellular reactions, despite that its dimension makes it an unlikely candidate to cluster heparan sulfate chains.

Mesenchymal cells use syndecans to adhere to ADAM 12 and β1 integrins to induce spreading (36, 40). The report by Iba *et al* describes a novel finding in cell adhesion whereby a syndecan is the primary receptor, but requires β1 integrins for cell spreading and stress fiber formation. This is in contrast to the more extensively studied adhesion to fibronectin, where β1 integrins are the primary receptor but syndecans are also required for full spreading and cytoskeletal reorganization (14). We now show that this process can be induced by a short HS-binding peptide alone.

The presence of the peptide in the tissue may be regulated by the cells. Chondroadherin exists in two forms in cartilage, where one form represents a cleavage product that lacks the last nine amino acids (20) constituting most of the peptide that we now studied. Whether this cleavage is regulated, and if so how is not known, but different cartilages appear to contain different relative proportions (41). Another issue is whether the peptide can be released to stimulate the cells or if this is accomplished by a longer fragment or the full length protein containing the peptide sequence.

It is relevant to speculate that the peptide fragment generated after cleavage plays an important role in the tissue through feedback regulation to the cell. The presented experiments show several novel findings. It appears that the detailed structure of the syndecan side chains and their interactions vary between the family members even on a homogenous cell line. The specific interacting domain of the glycosaminoglycans is likely to represent only a few disaccharide repeats where the chondroadherin peptide would represent a stretch of some 40 Å, provided that it is extended, which matches the expected length of the glycosaminoglycan binding domain. A peptide longer than the presently studied one will extend over a longer sequence of disaccharides with an ensuing contribution by more, some weak, interactions. An effect will be binding expanded to include a larger number of variants of the disaccharide repeat structure. This is likely to lead to decreased specificity for the binding. Signals elicited by the interaction of the heparan sulfate chain are clearly sufficient to induce changes of the cytoskeleton and enhance the presentation and/or activity of the integrins.

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Footnotes

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FIGURE LEGENDS

FIGURE 1. **Chondroadherin binding to heparin.**
Recombinant chondroadherin (30 μg) expressed in EBNA-cells and the *hbd*-CKFPTKRSKKAGRH^{359} (30 μg) respectively, were passed over a heparin-Sepharose column (Pharmacia). The column (1 ml) was washed with 5 mM sodium phosphate, 0.075 M NaCl, pH 7.4 and eluted with a gradient of 0.075 M- 1 M NaCl in 5 mM sodium phosphate, pH 7.4. Recombinant chondroadherin eluted with the peak at 0.6 M NaCl, while the peptide showed higher affinity eluting at 0.8 M NaCl (insert). The SDS-PAGE shows the preparation of chondroadherin applied to the heparin column (inserted).

FIGURE 2. **Binding of the heparin binding peptide CKFPTKRSKKAGRH^{359} to cells demonstrated by FACS.**
A. 105kc cells were suspended in PBS, 0.5% BSA and incubated on ice in the presence of *the hbd* biotin-CKFPTKRSKKAGRH^{359} in complex with Alexa Fluor 488 conjugated streptavidin (streptavidin-488) (▬▬) or streptavidin-488 (——) and analyzed for binding by FACS analysis.
B. 105kc cells cultured in medium with or without sodium chlorate present were detached and incubated on ice in the presence of the *hbd* biotin-CKFPTKRSKKAGRH^{359} in complex with streptavidin-488 (▬▬) or streptavidin-488 (——) and analyzed for binding by FACS analysis. *Insert:* Autoradiography of 105kc cell cultured with or without sodium chlorate in presence of ^{35}S-sulfate.

FIGURE 3. **Binding of the hbd-CKFPTKRSKKAGRH^{359} and the variant peptide CRFPTRKSRRAGKH to cells demonstrated by FACS.**
105kc cells were suspended in PBS, 0.5% BSA and incubated on ice in presence of the *hbd*- biotin-CKFPTKRSKKAGRH^{359} (A) or biotinylated control variant peptide CRFPTRKSRRAGKH (B) at 2.5, 5 and 10 μM in complex with streptavidin-488 and analyzed for binding by FACS.

FIGURE 4. **Chondroadherin binding to membrane proteoglycans on 105kc cells.**
Biotin-labeled membrane proteins (from 20x10^6 cells) were passed over a control-agarose column followed by a chondroadherin-agarose column (Mini Leak). The control (1, 2, 5, 6) and chondroadherin (3, 4, 7, 8) columns were washed and eluted with 10 mM EDTA containing 6 M urea (lane 1, 3, 5, 7)

and then 0.5 M NaCl (lane 2, 4, 6, 8). Eluted material was precipitated with ethanol and separated on a 4-12 % SDS-polyacrylamide gel, electro-transferred to a PVDF membrane and detected by western blotting before (lane 5-8) or after heparitinase and chondroitinase ABC treatment (lane 1-4). Biotin-labeled proteins were detected using streptavidin HRP and the ECL system.

FIGURE 5. **FACS analysis to determine the presence of syndecans at the human chondrosarcoma 105kc cell surface.**
A. 105kc cells ($0.1x10^6$) were suspended in PBS, 0.5% BSA and labeled with either syndecan 1 (---) or syndecan 2 (---) specific antibodies and detected by an Alexa Fluor 488 conjugated sheep anti-mouse antibody.
B. 105kc cells ($0.1x10^6$) were suspended in PBS, 0.5% BSA and labeled with either syndecan 3 (---) or syndecan 4 (---) specific antibodies and detected by a FITC conjugated goat anti-rabbit antibody.

FIGURE 6. **Affinity of cell surface proteoglycans to chondroadherin and the hbd-CKFPTKRSKKAGRH^{359} peptide from human chondrosarcoma 105kc cells.**
A. Membrane proteins (from $5x10^6$ 105 KC cells) were digested with heparitinase and separated by SDS-PAGE (14-12 % gel), transferred to a PVDF membrane, incubated with the pan-syndecan antibody (or syndecan 4 specific antibody, inserted) and detected using an anti-rabbit HRP antibody and the ECL system. The arrows indicate the expected positions of the core proteins of the four purified syndecans.
B. Biotin labeled membrane proteins (from $5x10^6$ cells) were passed over a control-agarose column followed by a chondroadherin-agarose column. Bound material was eluted with 0.5 M NaCl and eluted proteoglycans were immunoprecipitated with using a pan -syndecan antibody. Immunoprecipitated material was treated with heparitinase, separated by SDS-PAGE (10% gel), and transferred to a PVDF membrane labeled core proteins were detected using streptavidin HRP and the ECL system.
Lane 1 represents material eluted from the control-column and lane 2 material eluted from the chondroadherin-column.
C. Membrane proteins (from $5x10^6$ cells) were passed over a control column followed by the heparin binding peptide (immobilized to Ultra Link) column. Bound material was eluted with 0.5 M NaCl, treated with heparitinase, separated by SDS-PAGE (10% gel) and transferred to a PVDF membrane, followed by detection using a pan- syndecan antibody, and an anti rabbit HRP antibody using the ECL system. Lane 1 represents material eluted from the control-column and lane 2 material eluted from the peptide-column.

FIGURE 7. **Affinity of proteoglycans from MC3T3-E1 cells to the hbd-CKFPTKRSKKAGRH^{359} and their immunodetection.**
A. Protein extract from $5x10^6$ MC3T3-E1 cells, undigested (-) or digested with heparitinase (+), were separated by SDS-PAGE (10% gel), transferred to a PVDF membrane, incubated with the specific syndecan antibodies and detected using an anti-mouse HRP antibody and the ECL system.
B. Proteins extracted from $5x10^6$ MC3T3-E1 cells were passed over a control column and then over the heparin binding peptide (immobilized to Ultra Link) column. Bound material was eluted with 1.5 M NaCl. Eluted material was divided into two aliquots and one was treated with heparitinase. Undigested (-) and digested (+) material was separated by SDS-PAGE (10% gel) and transferred to a PVDF membrane. The membrane was probed using syndecan 1 and 4 specific antibodies, followed by an anti rabbit HRP antibody using the ECL system.

FIGURE 8. **Signaling elicited by binding of heparin ligand peptide.**
A. Tissue culture 6-well dishes (Costar) were coated overnight with 20 µM peptide. The dishes were blocked for nonspecific binding with 0.5% BSA. To determine ERK phosphorylation, human chondrocytes were serum starved for 48 hours prior to the experiment. Cells were added to the wells (500 000/well), coated with the peptide or to wells blocked with BSA only and allowed to adhere for 1 hour. All cells (bound and unbound) were lyzed and analyzed by linear (10%) SDS-polyacrylamide gel and western blotting. Blots were developed with the phosphorylation-specific antibody followed by visualization with chemiluminescence detection using the ECL system (Amersham Biotechnology). The membranes were re-probed with an antibody to determine total ERK1/ERK2. Gel-Pro Analyzer® was used for scanning of blots for quantification of phosphorylations.

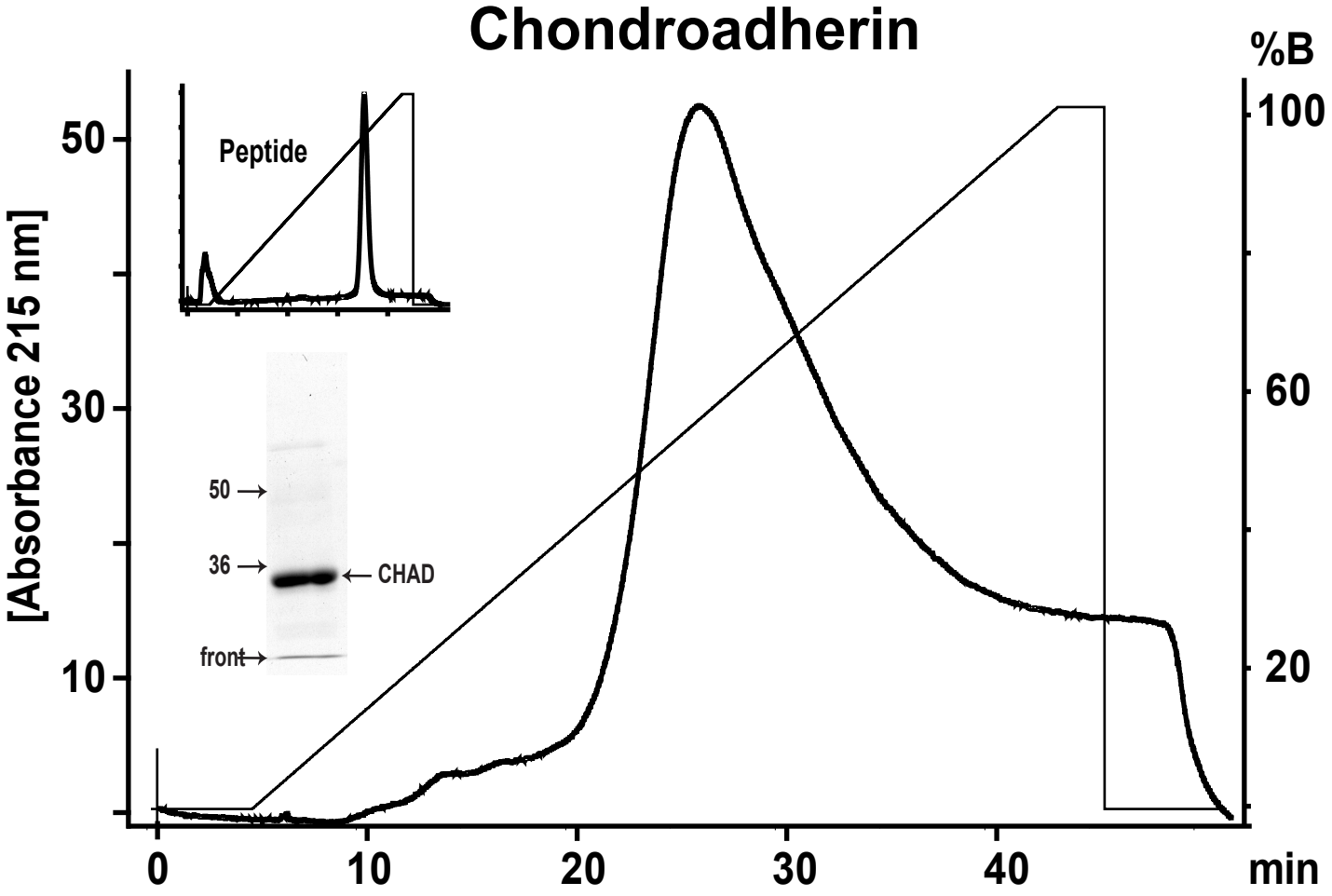
B. Tissue culture dishes were coated with 0.16, 0.6, 2.5 or 10 μM peptide and blocked for non-specific binding with BSA. The cells were suspended in PBS containing 0.1% BSA and 25 U/ml collagenase (CLSPA, Worthington) and added to the wells and allowed to adhere for 1 hour at 37°C. Non-adherent cells were removed by washing. Spreading was visualized by light microscopy.
C. Representative blots are shown to demonstrate the level of specificity in the experiments.

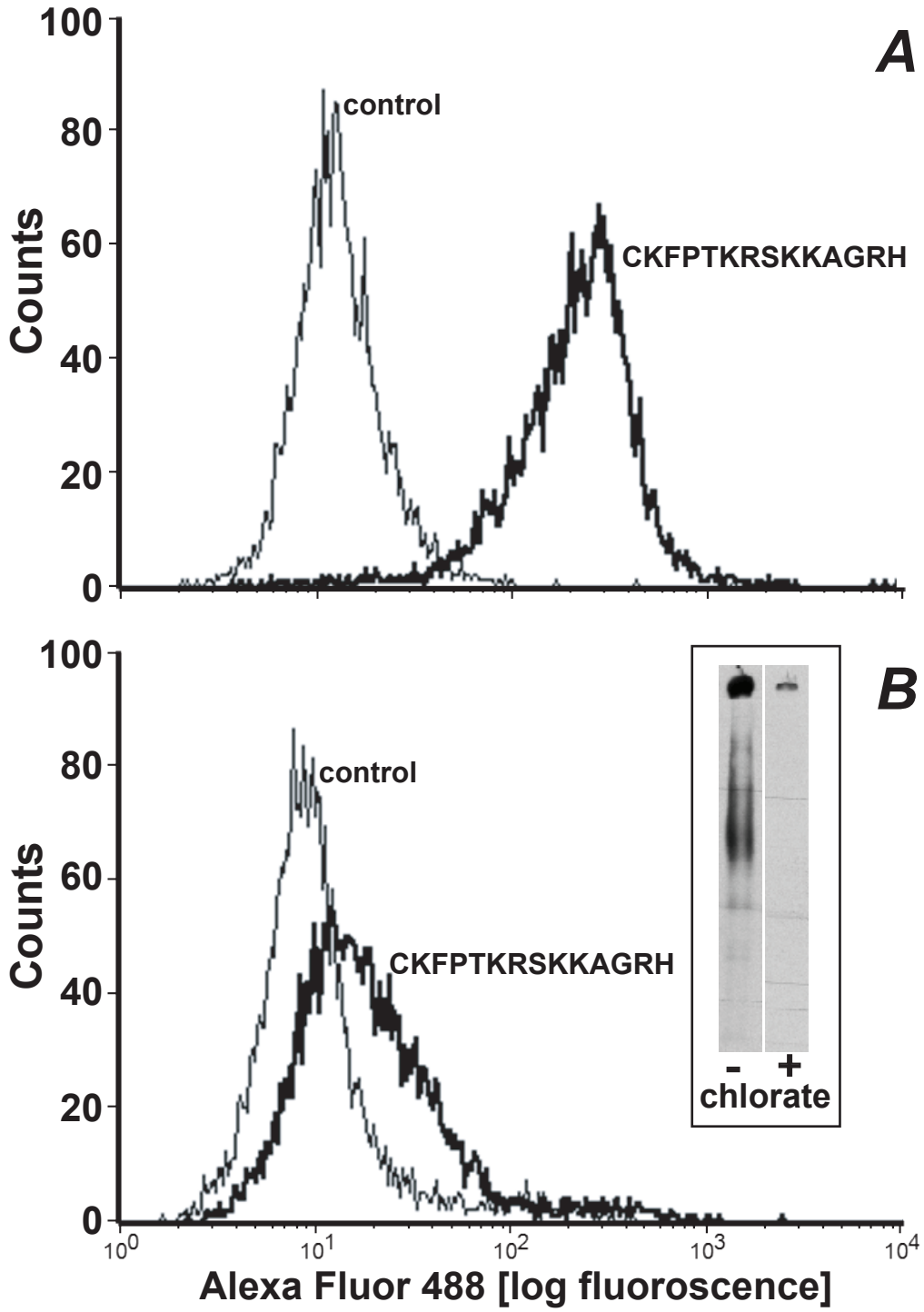
FIGURE 9. **Activation of signaling pathways and rearrangement of cytoskeletal elements upon exposure of cells to the hbd-CKFPTKRSKKAGRH^{359}**
A. *Tyrosine phosphorylation of signaling pathways in cells bound to chondroadherin upon addition of the hbd-CKFPTKRSKKAGRH^{359} .*
Tissue culture 6-well dishes (Costar) were coated overnight with 5 μg/ml chondroadherin. The dishes were blocked for nonspecific binding with 0.5% BSA. To determine ERK phosphorylation, primary human chondrocytes were serum starved and analyzed as described in figure 8A.
B. *Immunochemical staining of adhesion complexes.*
Chamber slides were coated with 5 μg/ml of CHAD (top and bottom panels) or 33 μg/ml (20 μM) of the *hbd*-CKFPTKRSKKAGRH^{359} peptide (middle panel) and blocked for nonspecific binding with BSA (0.5%). The cells (105kc) were allowed to adhere for 1 hour in the absence (top and middle panel) or in the presence (bottom panel) of the peptide (150 μM, 250 μg/ml) and adhesion complexes were identified using antibodies against Phalloidin (red) and vinculin (green).

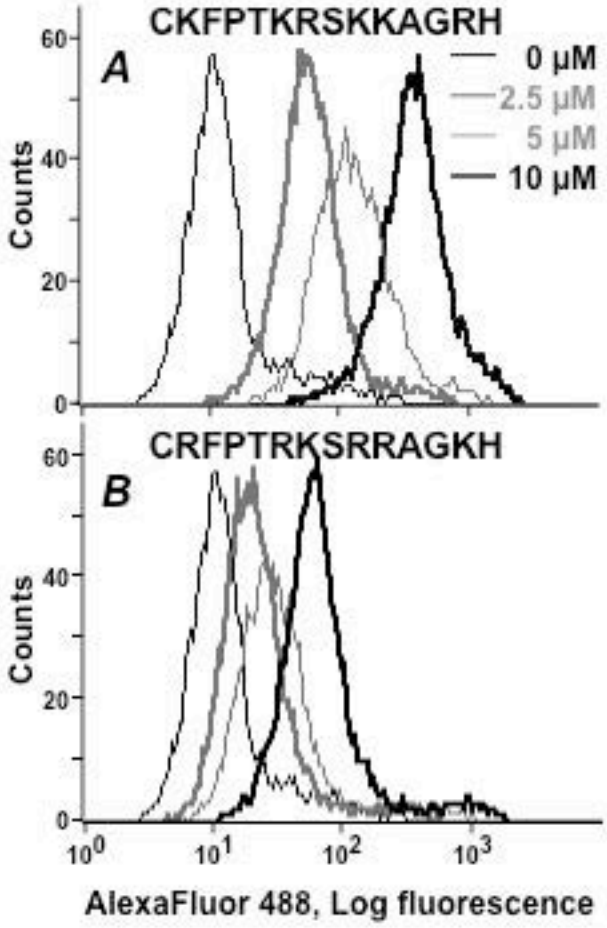
FIGURE 10. **Adhesion of human chondrosarcoma 105kc cells to human chondroadherin and collagen type II in the presence of the hbd-CKFPTKRSKKAGRH^{359} .**
48-well tissue culture dishes were coated with chondroadherin, expressed in E-coli (top part) or collagen type II (bottom part) at 0.04 or 5 μg/ml PBS, and blocked for non-specific binding with BSA. The cells were suspended in PBS, containing 0.1% BSA plus 25 U/ml collagenase and added to the wells in the absence or presence of synthetic peptide (150 μM, 250 μg/ml) and allowed to adhere for 1 hour at 37°C, see histograms. Non-adherent cells were removed by washing. Spreading was visualized by light microscopy, and adhesion was determined by analyses of lysosomal N-acetylglucosaminidase. Adhesion is expressed as number of cells, where the data represents the average of two wells. Independent experiments showed similar results.

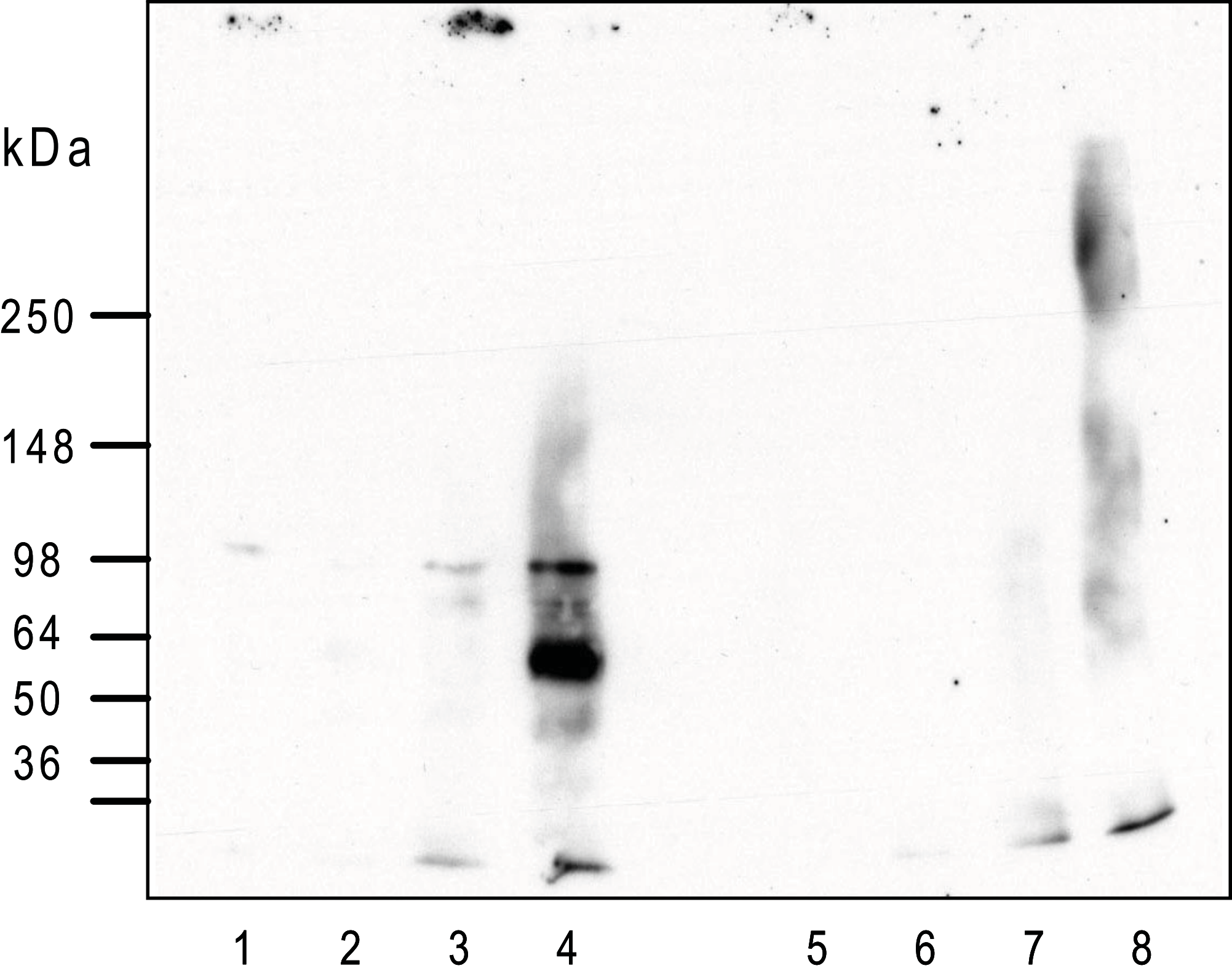
FIGURE 11. **Adhesion of primary bovine chondrocytes to human chondroadherin in the presence of the hbd-CKFPTKRSKKAGRH^{359} .**
Tissue culture dishes were coated with chondroadherin (5 μg/ml) expressed in E-coli and blocked for non-specific binding with BSA. The cells were suspended in PBS containing 0.1% BSA and 25 U/ml collagenase, added to the wells in the absence or presence of the synthetic *hbd*-CKFPTKRSKKAGRH^{359} (150 μM, 250 μg/ml) and allowed to adhere for 1 hour at 37 ºC. Non-adherent cells were removed by washing. Adhesion was determined by analyzing lysosomal N-acetylglucosaminidase. Adhesion is expressed as percentages of cells and the data represents the average of two wells. Independent experiments showed similar results.

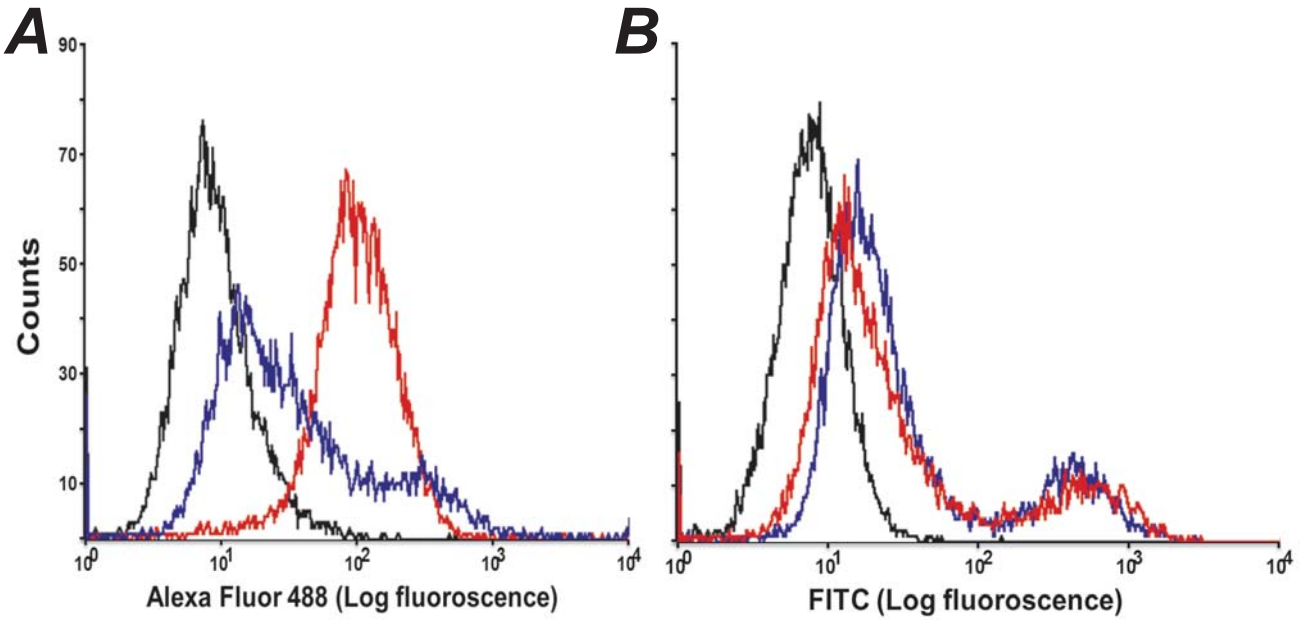
FIGURE 12. **The effect of glycosaminoglycans on cell interaction of the hbd-CKFPTKRSKKAGRH^{359} demonstrated by FACS.**
The *hbd-biotin* CKFPTKRSKKAGRH^{359} in complex with streptavidin-488 were pre-incubated with heparan sulfate, heparin, Chondroitin Sulfate A (CS-A), dermatan sulfate (CS-B), chondroitin sulfate C (CS-C) or keratan sulfate at different concentrations (100, 20, 4, 0.8 and 0.16 μg/ml PBS, 0.5% BSA). The human chondrosarcoma cells were incubated with the pre-incubated mixtures on ice and washed twice prior analysis by FACS.
A: FACS analysis chromatogram of heparan sulfate and CS-C.
B: FACS analysis of all glycosaminoglycans presented as fluoresces intensity.


Chondroadherin
%B
100
60
20
[Absorbance 215 nm]
50
30
10
Peptide
50
36
CHAD
front
0
10
20
30
40
min


A
100
80
60
40
20
0
Counts
control
CKFPTKRSKKAGRH
B
100
80
60
40
20
0
Counts
control
CKFPTKRSKKAGRH
- +
chlorate
10^0
10^1
10^2
10^3
10^4
Alexa Fluor 488 [log fluoroscence]

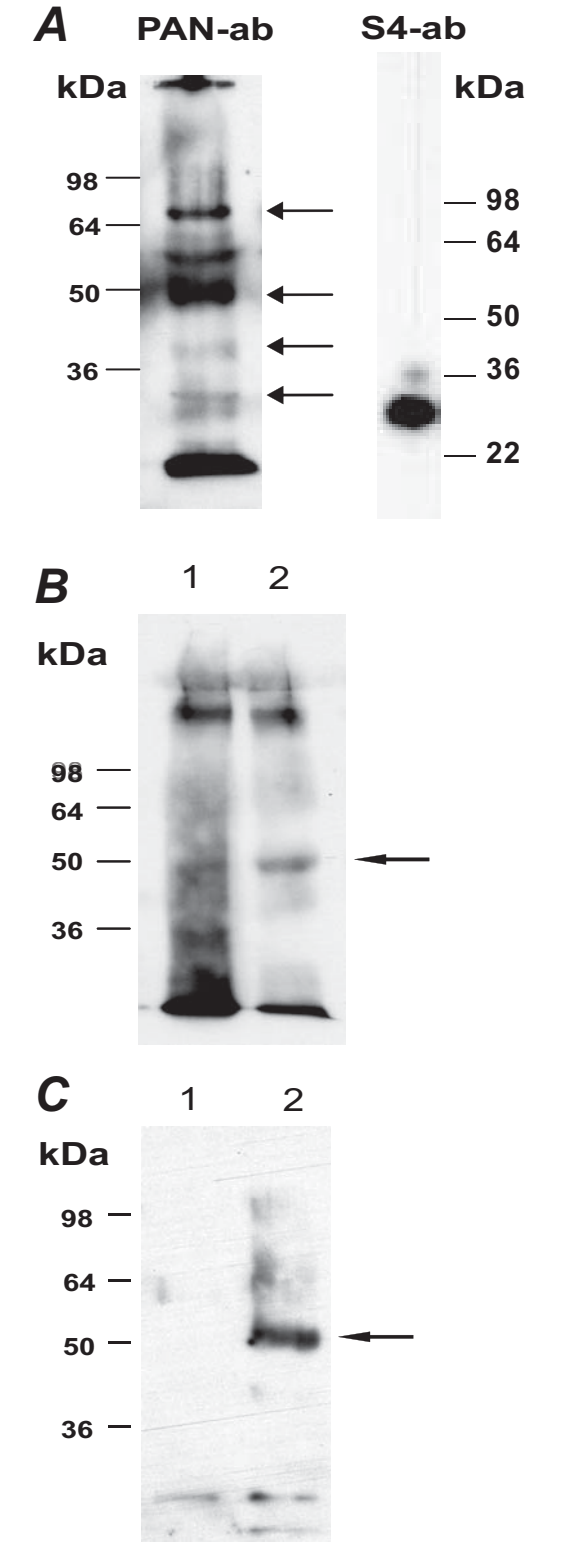

CKFPTKRSKKAGRH
A
0 µM
2.5 µM
5 µM
10 µM
Counts
60
40
20
0
CRFPTRKSRRAGKH
B
Counts
60
40
20
0
10^0
10^1
10^2
10^3
AlexaFluor 488, Log fluorescence

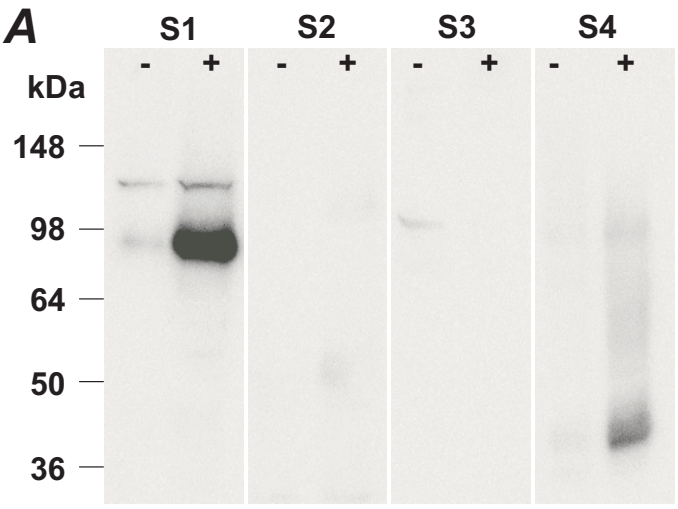

kDa
250
148
98
64
50
36
1
2
3
4
5
6
7
8

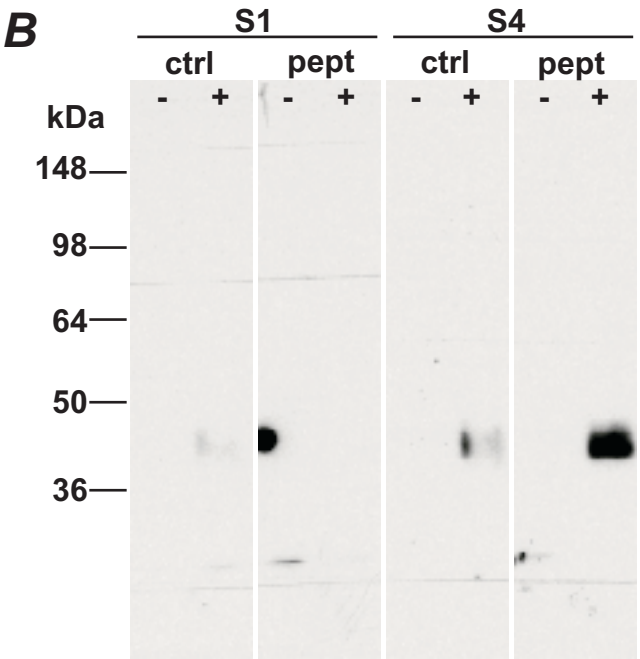


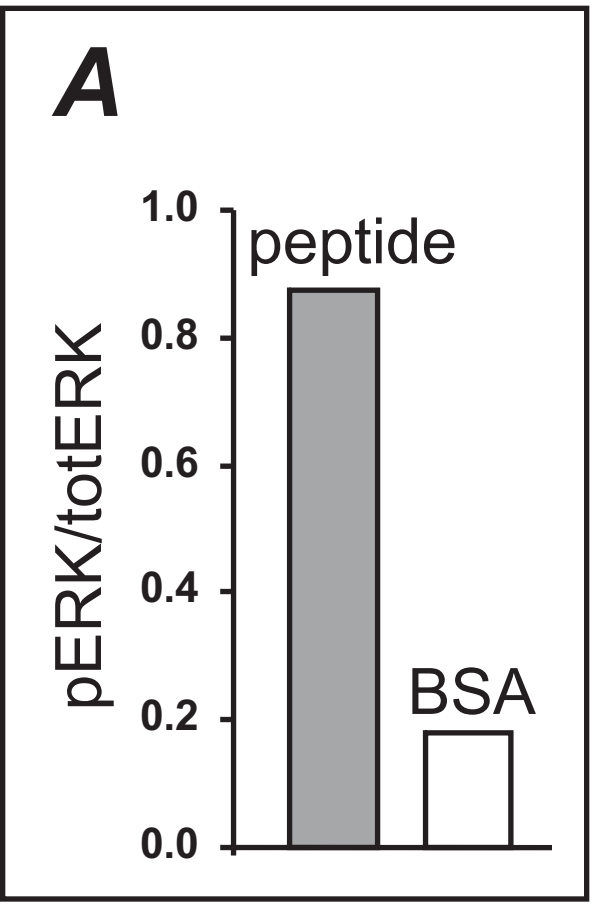
	Ctrl-anti mouse	Sdc1 (Red)	Sdc2 (Blue)
Geomean	8.2	87.6	30.2

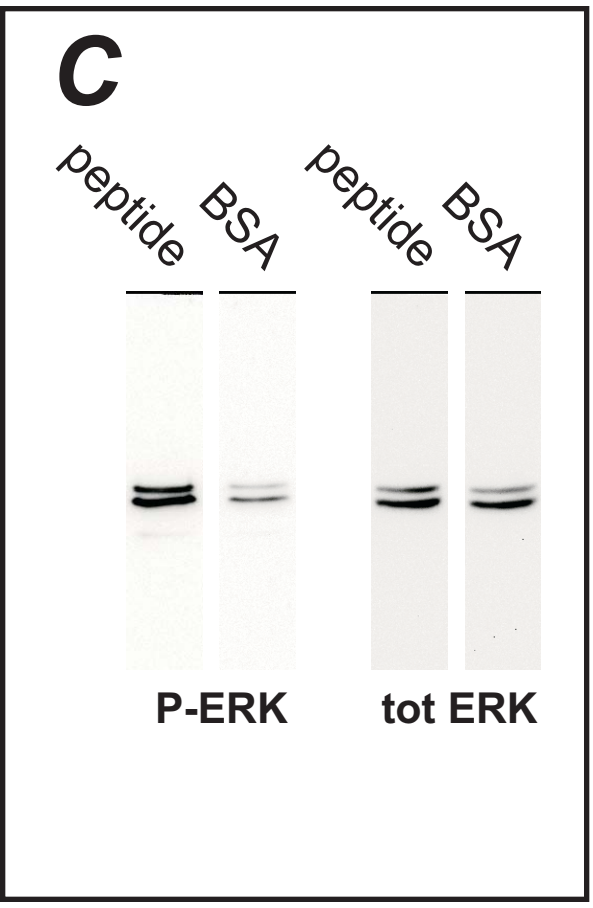
	Ctrl-anti rabbit	Sdc3 (Red)	Sdc4 (Blue)
Geomean	7.6	24.8	28.9

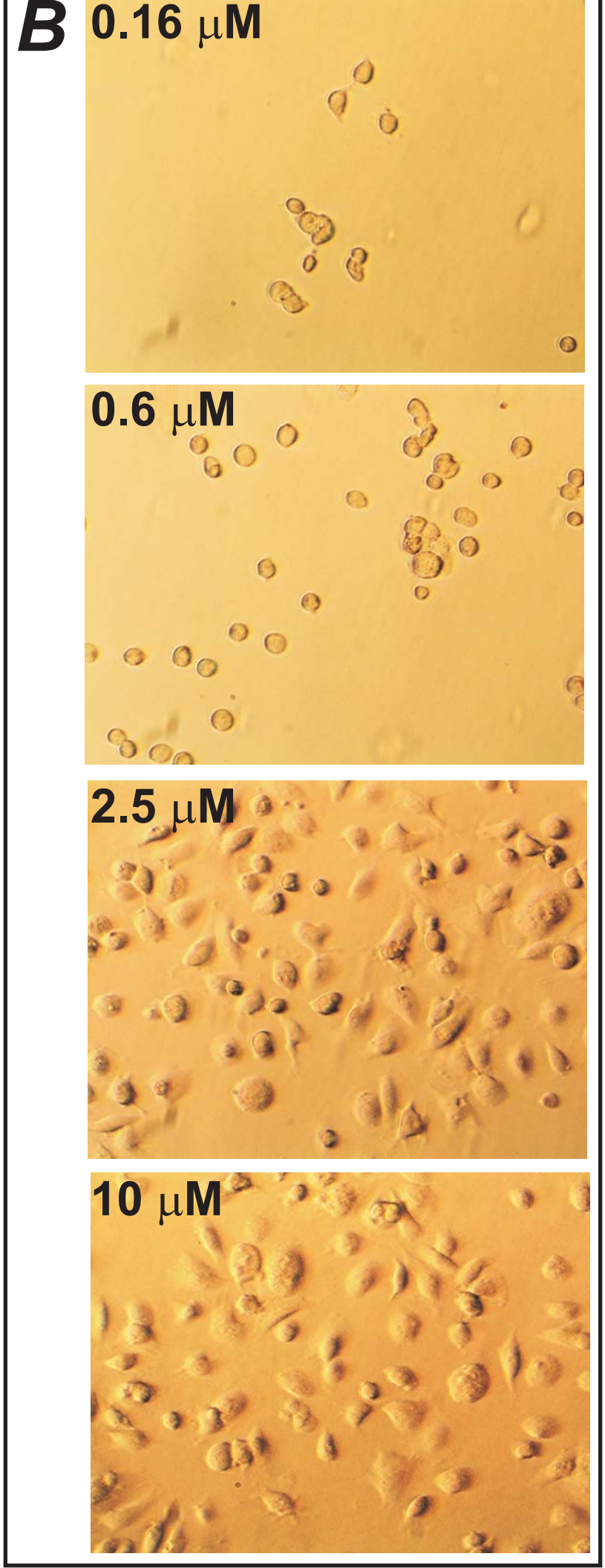

A
PAN-ab
S4-ab
kDa
98
64
50
36
kDa
98
64
50
36
22
B
1
2
kDa
98
64
50
36
C
1
2
kDa
98
64
50
36

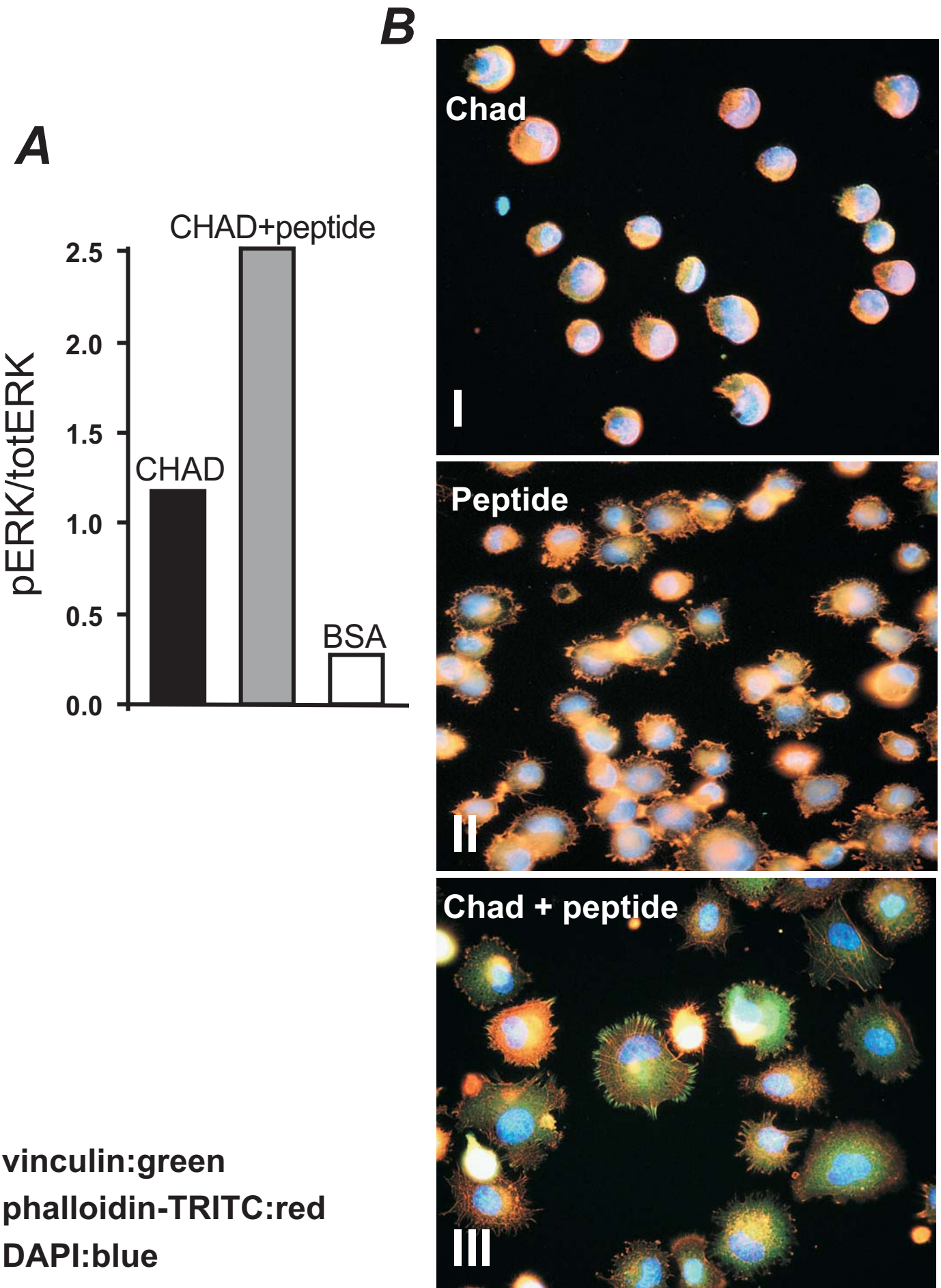

A
S1
S2
S3
S4
- + - + - + - +
kDa
148
98
64
50
36

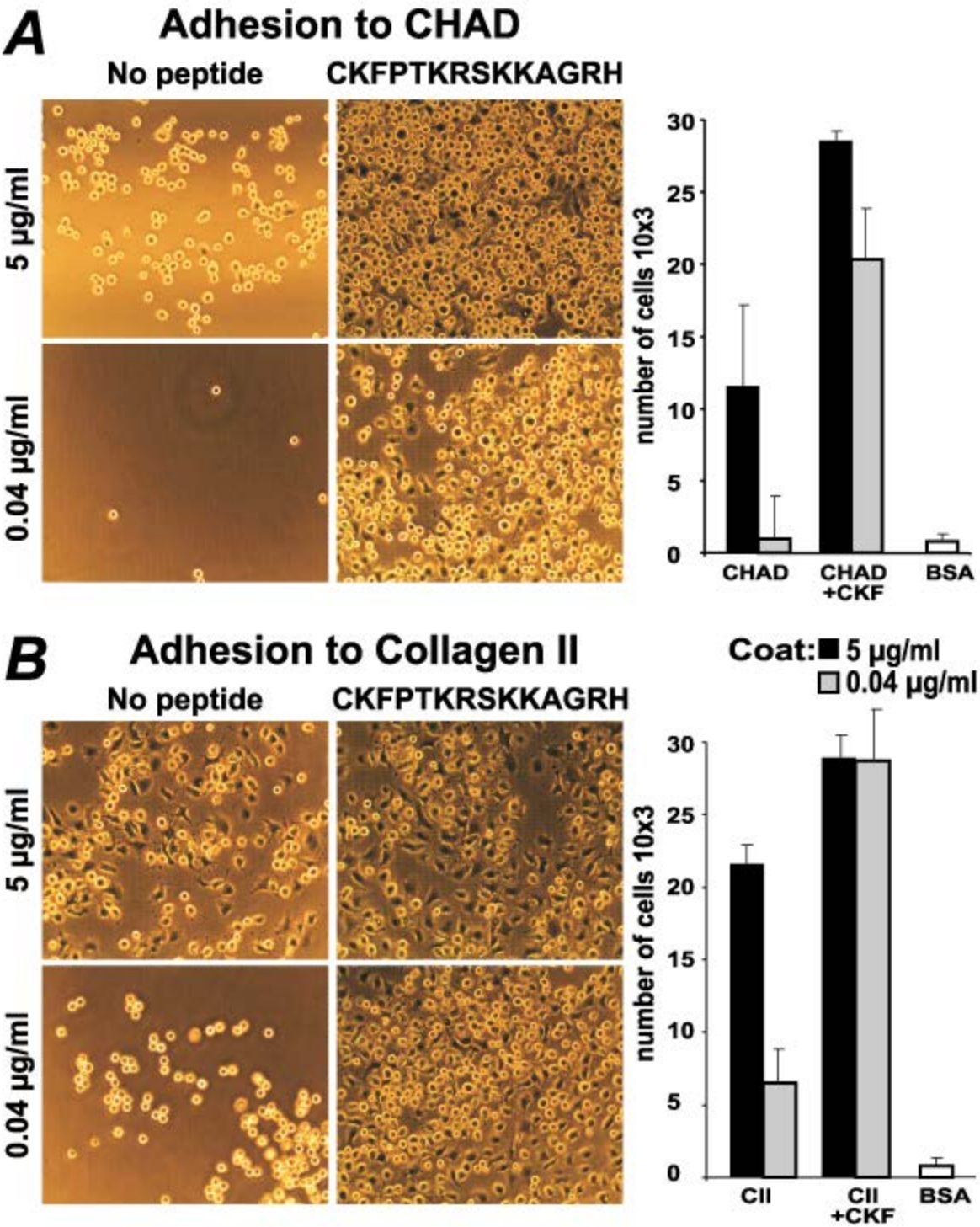

B
S1
S4
ctrl
pept
ctrl
pept
- + - + - + - +
kDa
148
98
64
50
36

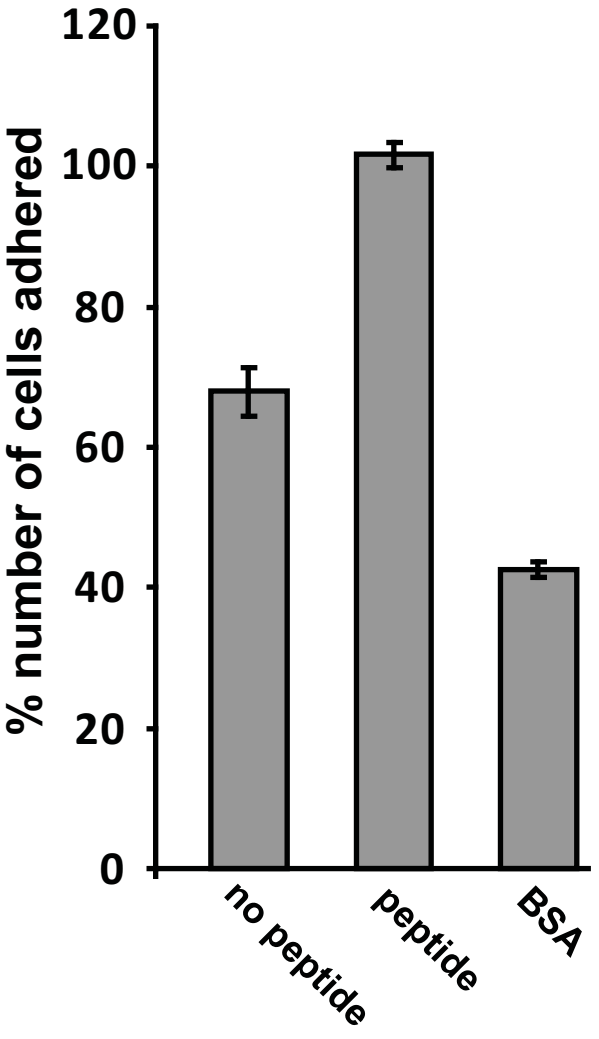

A
1.0
0.8
0.6
0.4
0.2
0.0
pERK/totERK
peptide
BSA

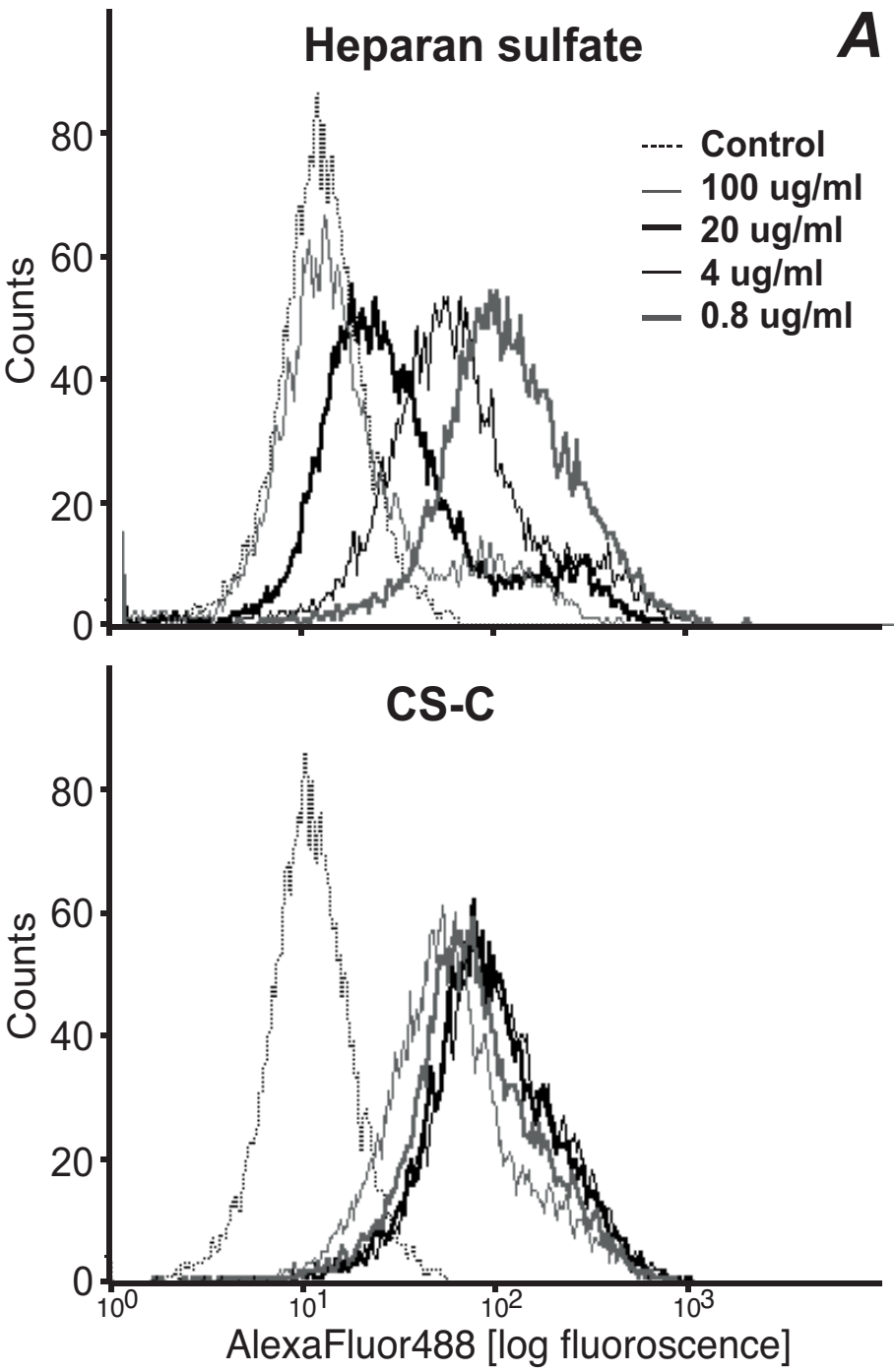

C
peptide
BSA
peptide
BSA
P-ERK
tot ERK

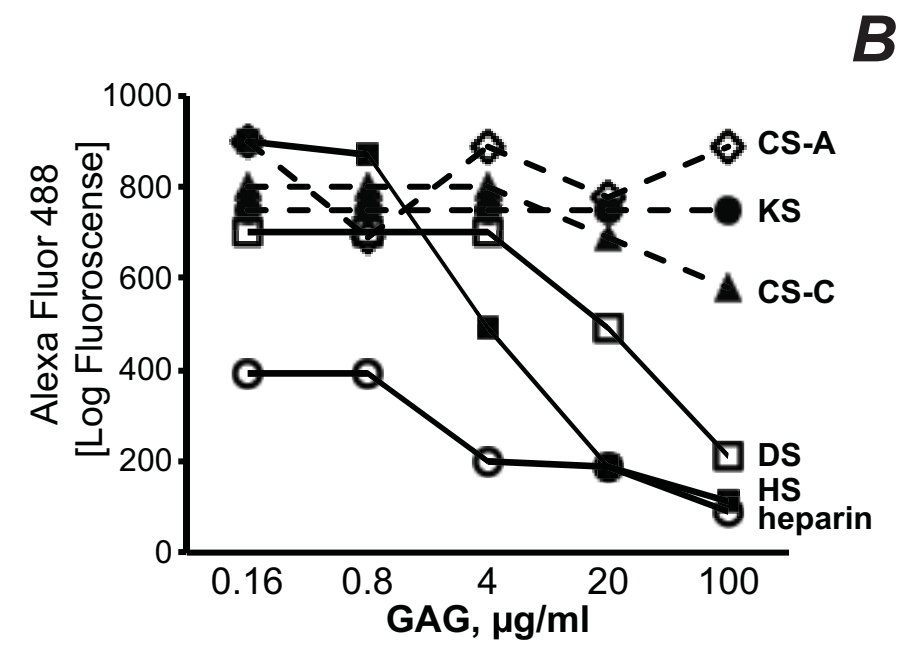

B
0.16 μM
0.6 μM
2.5 μM
10 μM


A
CHAD+peptide
2.5
2.0
1.5
1.0
0.5
0.0
pERK/totERK
CHAD
BSA
B
Chad
I
Peptide
II
Chad + peptide
III
vinculin:green
phalloidin-TRITC:red
DAPI:blue


A
Adhesion to CHAD
No peptide
CKFPTKRSKKAGRH
5 µg/ml
0.04 µg/ml
number of cells 10x3
30
25
20
15
10
5
0
CHAD
CHAD +CKF
BSA
B
Adhesion to Collagen II
Coat: 5 µg/ml
0.04 µg/ml
No peptide
CKFPTKRSKKAGRH
5 µg/ml
0.04 µg/ml
number of cells 10x3
30
25
20
15
10
5
0
CII
CII +CKF
BSA


% number of cells adhered
120
100
80
60
40
20
0
no peptide
peptide
BSA


A
Heparan sulfate
Control
100 ug/ml
20 ug/ml
4 ug/ml
0.8 ug/ml
Counts
0
20
40
60
80
CS-C
Counts
0
20
40
60
80
10^0
10^1
10^2
10^3
AlexaFluor488 [log fluoroscence]


B
Alexa Fluor 488
[Log Fluoroscense]
0
200
400
600
800
1000
CS-A
KS
CS-C
DS
HS
heparin
0.16
0.8
4
20
100
GAG, µg/ml