Annexin 6 is a putative cell surface receptor for chondroitin sulfate chains

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Summary

Chondroitin sulfate proteoglycans, including PG-M/versican, inhibit cell-substratum adhesion. They achieve this through their chondroitin sulfate chains. In order to define the molecular mechanism for this inhibition, we investigated the influence of these chains on cell attachment to substratum, the first step in cell adhesion. Chondroitin sulfate chains did not prevent cell attachment. In fact, a variety of cells attached to chondroitin sulfate, implying the existence of putative receptors and/or binding proteins for this extracellular matrix glycosaminoglycan. Detergent-extracted human fibroblast membrane protein extracts were examined by affinity chromatography in the presence of Ca^{2+} on chondroitin sulfate immobilized on agarose CL-6B. A 68 kDa and a 35 kDa protein were isolated, sequenced and demonstrated to be annexin 6 and annexin 4, respectively. Next we used A431 cells devoid of annexin 6 expression to verify that annexin 6 is the receptor for this glycosaminoglycan. We confirmed that A431 cells were unable to attach to the chondroitin sulfate substratum and that the stable transfectants expressing annexin 6 conferred the ability to attach to chondroitin sulfate chains. Further, the presence of annexin 6 on the cell surface was confirmed by fluorescence-activated cell sorting analysis using the annexin 6 antibody; annexin 4 is not present on the cell surface. In summary, annexin 6 is a candidate receptor for chondroitin sulfate chains.

Key words: Anti-adhesion, Chondroitin-sulfate-binding proteins, Cell attachment, CSPE, PG-M/versican

Introduction

Most extracellular matrix molecules promote cell adhesion, modify cytoskeletal organization and play important roles in regulating a variety of cellular activities, such as cell growth, gene expression and cell differentiation. However, there are a group of extracellular matrix molecules that inhibit cell adhesion (anti-adhesive molecules). Members of this group include tenascin, thrombospondin, laminin, mucin, SPARC and chondroitin sulfate (CS) proteoglycans (Murphy-Ullrich, 1995; Chiquet-Ehrismann, 1995; Murphy-Ullrich, 2001). Receptors have been identified for some of these anti-adhesive molecules and attempts have been made to characterize their mechanism. Expression of these molecules is spatiotemporally regulated during development and regeneration (through the regulation of cell-matrix interactions and cell shape), which suggests that the anti-adhesive molecules are critical for these events.

PG-M/versican, a large CS proteoglycan, is expressed preferentially in the mesenchymal cell condensation area of chick limb bud at the prechondrogenic stage (Kimata et al., 1986; Shinomura et al., 1990). Almost all CS proteoglycans inhibit cell-substratum adhesion in vitro (Rich et al., 1981; Brennan et al., 1983; Lewandowska et al., 1987; Yamagata et al., 1989; Winnemoller et al., 1991; Bidanset et al., 1992). In addition, immunohistochemical detection of CS chains revealed that they are localized at the cell surface and are excluded from focal contacts of cultured fibroblasts (Yamagata et al., 1993). Moreover, the reduction of the PG-M/versican level by anti-sense RNA expression in osteosarcoma cells MG63, which express large quantities of PG-M/versican, resulted in an increase in cell adhesion, the formation of focal contacts and the elaboration of stress fibers. This supports the notion that PG-M/versican, which is present in the extracellular matrix near the cell surface, functions as an anti-adhesive molecule in many cell types (Yamagata and Kimata, 1994). We previously showed that PG-M/versican, when immobilized on tissue culture substrata, had a marked inhibitory effect on the adhesion of cells to precoated proteins (Yamagata et al., 1989). The inhibitory activity of CS proteoglycan was abolished by chondroitinase digestion, and CS-conjugated serum albumin also inhibited cell adhesion. These results suggest that the inhibitory activity is caused by CS chains immobilized on the tissue culture plastic wells through their protein portions and is independent of the core protein of proteoglycan. To investigate this suggestion, we developed new molecules, L-α-dipalmitoylphosphatidylethanolamine (PE)-derivatized glycosaminoglycans (GAGs) (GAG-PEs), by chemically coupling the reducing terminals of GAGs to the amino group of PE, which enables hydrophilic GAGs to bind to hydrophobic tissue culture wells like native chondroitin sulfate proteoglycans do. In fact, CS-derivatized PE (CSPE) mimics the inhibitory activity of CS proteoglycans (Sugiura et al., 1993). These results support the idea that immobilized or matrix-associated CS chains inhibit cell-substratum adhesion, whereas free CS chains do not. They also indicate that this new molecule is a novel tool for examining the effect of CS chains on cell adhesion.

Annexins are a family of proteins that have the ability to bind to acidic phospholipids in the presence of Ca^{2+}. All annexins possess variable N-terminal domains followed by conserved core regions that impart membrane-binding capabilities and usually contain four 70-80 amino-acid repeats with an annexin consensus sequence. The core region of annexin 6, however, contains eight such repeats. These conserved core regions are responsible for the Ca^{2+}-dependent binding of the proteins to phospholipids. By contrast, the N-terminal domains of the annexins are highly variable and may contribute to the specific functions of the various annexins (Raynal and Pollard, 1994; Edwards and Moss, 1995). Some of annexins bind to GAG in a Ca^{2+}-dependent manner. Annexin 2 has specific and high-affinity heparin-binding activity (Kassam et al., 1997). Annexin 4 binds to heparin, heparan sulfate and CS columns in a Ca^{2+}-dependent manner, annexin 5 to heparin and heparan sulfate columns in a Ca^{2+}-dependent manner and annexin 6 to heparin and heparan sulfate columns in a Ca^{2+}-independent manner and to CS columns in a Ca^{2+}-dependent manner (Ishitsuka et al., 1998). These results suggest that some annexin species may function as recognition elements for GAGs under some conditions.

In this study, we have further investigated the mechanism of anti-adhesive activity of CS chains. We first tested the possibility that a cell surface receptor for CS chains is involved in this activity using a cell attachment assay. We have successfully isolated a 68 kDa protein as a candidate receptor for CS chains and identified that protein as annexin 6. Moreover, taking advantage of A431 cells that do not express annexin 6 and transfecting them with exogenous annexin 6, we have demonstrated that annexin 6 is directly involved in the attachment of cells to CS chains and is expressed on cell surfaces.

Materials and Methods

Materials

The GRGDSP and GRGESP peptides were purchased from GIBCO. CS (shark) conjugated to L-α-dipalmitoylphosphatidylethanolamine (CSPE), prepared as described previously (Sugiura et al., 1993), was donated by Sugiura from our laboratory. CS from shark cartilage, CS from whale cartilage, dermatan sulfate from pig skin, heparin, chondroitin (a chemically desulfated derivative of whale cartilage chondroitin sulfate A) and hyaluronan were purchased from Seikagaku Corp., Tokyo. CS-conjugated Sepharose CL-6B gels were prepared by the method reported previously with a minor modification (Funahashi et al., 1982). Heparin-conjugated Sepharose Cl-6B gels were purchased from Amersham Pharmacia Biotech. Anti-annexin 6 rabbit polyclonal antibodies were prepared by the affinity purification of antiserum against annexin 6 purified from bovine liver (Ishitsuka et al., 1998). The purified antibodies also recognize human annexin 6 and are only reactive to this molecule when in whole cell lysate (data not shown). Anti-human annexin 6 mouse monoclonal antibody was purchased from Transduction Laboratories. Anti-hemagglutinin A tag rabbit polyclonal antibody was from Clontech, CA. Anti-human annexin 4 mouse monoclonal antibody was from Transduction Laboratories, KY, and from ZYMED laboratories, CA. Rabbit anti-focal adhesion kinase (FAK), rabbit anti-β-1 integrin and goat anti-human annexin 4 polyclonal antibodies were from Santa Cruz Biotechnology. FITC-conjugated anti-rabbit and anti-goat IgG were from Molecular Probes. Propidium iodide was from Nakarai Tesque, Kyoto.

Cell and cell culture

Human osteosarcoma MG63 cells, human fibroblast IMR90, WI38, MRC5 and human epidermoid carcinoma cell A431 cells were obtained from Japanese Cancer Research Resources Bank (JCRB)-Cell, Tokyo, Japan. Chick embryonic fibroblasts were established from 10-day-old chick embryos as described previously (Yamagata et al., 1986). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences), 50 units/ml penicillin and 50 μg/ml streptomycin.

The cell attachment assay

The centrifugation assay described previously (Friedlander et al., 1988; Ernst et al., 1995) was modified, and the procedure is summarized in Fig. 1A. U-shaped wells in a 96-well polystyrene plates (Nunc-Immuno plate U) were coated with 100 μl of 5 μg/ml human fibronectin (FN) (Gibco), 8 μg/ml concanavalin A (ConA) (Gibco) or 5 μg/ml CS conjugated to L-α-dipalmitoylphosphatidyl-ethanolamine (CSPE) at 4°C overnight. The wells were washed three times with PBS (–) and were blocked with 10 mg/ml heated BSA in PBS (–) for 30 minutes. Cells cultured up to subconfluency were rinsed twice with PBS (–) and harvested by treating cells with 0.05% trypsin in PBS (–) at 37°C for 3 minutes. The cells were collected and washed twice with 1mg/ml trypsin inhibitor in PBS (–) and resuspended with the growth medium at 37°C for 10 minutes to allow cells to recover. Cells were then washed once with serum-free DMEM and suspended in the same medium. A portion of the cell suspension (5×10^3 cells/100 μl) was added into each well and incubated for 10 minutes. The plate was then centrifuged at 200 ***g*** for 1 minute. Each well was photographed under a microscope. On a non-adhesive substratum such as BSA, the centrifugal force predominates, so that the cells form a pellet at the bottom of the well. When cells attach to the substratum, cells bind in a uniform distribution both on the sides and at the bottom of the well. At the intermediate level of attachment, cells distribute in a ring, and the size of the ring depends upon the balance between the centrifugal force and the adhesion. For the assessment of the cell attachment activity, cells on each photograph were counted from the center of the well and the areas with 4×10^2 cells were determined. Values were obtained in triplicate independent experiments. From these values, we used an 'attachment index' to quantify the cell-substratum attachment using the following calculation: attachment index=(Δsubstratum–ΔBSA)/ΔBSA, where Δsubstratum and ΔBSA are the areas when a test sample and BSA were used as a substratum, respectively.

Purification and identification of receptor molecules for chondroitin sulfate chains

Human fibroblast WI38 cells and chick embryonic fibroblasts were cultured in 150 mm culture dishes (Falcon). Cells from 20 dishes were rinsed twice with ice-cold PBS (–) and harvested with a cell scraper. Cells were collected by centrifugation. The pellet was suspended in 0.25 M sucrose, 0.5 mM $CaCl_2$, 0.02 M Tris-acetate, pH 7.2, and gently homogenized with a glass-teflon homogenizer at 4°C. The homogenate was centrifuged at 2,000 ***g*** at 4°C for 10 minutes. The supernatant solution was centrifuged at 105,000 ***g*** at 4°C for 1 hour. The precipitate was obtained as the membrane fraction, and the membrane fraction was extracted with TNE buffer (10 mM Tris-HCl, pH 7.8, 1% NP-40, 0.15 M NaCl, 10 mM EDTA, 1 mM PMSF, 1 mg/ml leupeptin) at 4°C for 1 hour. The extract was centrifuged at 10,000 ***g*** for 25 minutes to remove the debris and then subjected to PD-10 (Amersham Pharmacia Biotech.), which was equilibrated with the affinity buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 1 mM PMSF, 1 mg/ml leupeptin, 10 mM β-mercaptoethanol, 5 mM $CaCl_2$, 5 mM $MgCl_2$). The sample in the affinity buffer (3 ml) was subjected to the CS-conjugated Sepharose CL-6B gel column.

After the column was washed with 20 ml of the affinity buffer, the bound proteins containing the expected receptor molecules for CS chains were eluted with the elution buffer (20 mM Tri-HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 5 mM EDTA, 1 mM PMSF, 1 mg/ml leupeptin). Subsequently, further elution was performed with 4 M urea. The eluted proteins were precipitated with trichloroacetic acid, dissolved in SDS-buffer and subjected to SDS-PAGE. The protein bands on the gel were digested with V8 protease and then subjected to the second SDS-PAGE. Some of the peptide bands were transferred to PVDF membrane and subjected to the Applied Biosystems model 476A sequencer and Applied Biosystems model 120A HPLC apparatus as described previously (Zhao et al., 1995).

Construction of human annexin 6 and the deletion mutants

Full-length human annexin 6 cDNA was obtained by RT-PCR using the sequence data (Crompton et al., 1988). Total cellular RNA was isolated from IMR90 cells using the Trizol reagent (Life Technologies). The first strand of cDNA was prepared using Ready-to-Go T-Primed First-Strand kit (Amersham Pharmacia biotech). A human annexin 6 PCR fragment was amplified by 25 cycles using Pfu DNA polymerase (Stratagene) and the following primers: forward1 5′-TGCGTCCGTCTGCGACCCGAG-3′ (corresponding to the position of –70 to –30 in human annexin 6 cDNA); reverse5 5′-GCGTTTCCTAAGCTCCACTGAAG-3′ (corresponding to the position of 2157 to 2179 in human annexin cDNA). The full-length cDNA was directionally inserted into the *Eco*RV site of pIRES1neo (Clontech Lab., Inc. USA). The deletion mutants of human annexin 6 were created by 20 cycles using Pfu DNA polymerase and the primers indicated in Fig. 6A. Wild-type (full-length human annexin 6) was amplified using the set of primers, foward1 and reverse 4:5′-TTAAG-CATAATCTGGAACATCATATGGATAGTCCTCACCACCACAGA-GAG-3′ (this primer contains the sequence corresponding to a hemagglutinin A epitope, stop codon, and the sequence corresponding to the amino acid sequence of ALCGGED of human annexin 6). ALT (the alternative splicing form) was created by combining the following two DNA fragments: the DNA fragment that was amplified with forward1 and reverse3 primer, 5′-CTGGGCATCTTCCCGTGCCT-3′ (corresponding to the amino acid sequence of QAREDAQ) and the DNA fragment that was amplified with forward3, 5′-GAAATAGCAGACA-CACCTAG-3′ (corresponding to the amino acid sequence of EIADTPS) and reverse4 primer. N (the N-terminal annexin-6-specific domain and the N-terminal half of the annexin 6-core region) was amplified using forward1 and reverse2 primer, 5′-AGCAGCATCAT-CATCTCC-3′ (corresponding to the amino acid sequence of GDDDAA). C+ (the N-terminal annexin-6-specific domain and the C-terminal half of the annexin 6-core region) was created by combining the following two DNA fragments: the DNA fragment that was amplified with the set of forward1 and reverse1 primer, 5′-CCG-GTACTTGGCACCCTGTG-3′ (corresponding to the amino acid sequence of AQGAKYR) and the DNA fragment that was amplified with forward2, 5′-GGAACTGTGCGCCCAGCCAA-3′ (corresponding to the amino acid sequence of GTVRPA) and reverse4 primer. C– (the N-terminal annexin 6-specific domain and the C-terminal half without the alternative splicing domain) was created by replacing the *Bam*HI/*Eco*RI fragment of ALT with the *Bam*HI/*Eco*RI fragment of C+. Each cDNA fragment was directionally inserted into the *Eco*RV site of pIRES1neo.

Transfection of A431 cells with human annexin 6 cDNA and its deletion mutants

Exponentially growing A431 cells were cultured at 1×10^5 cells in 60 mm dishes for 24 hours and transfected with the cDNAs; they continued to be cultured for another 24 hours. Transfection was achieved using Trans IT polyamine transfection reagents (Pan Vera corp., WI). Geneticin-resistant colonies were isolated two weeks after the transfection and examined for the annexin 6 expression by western blotting. Control transfections (transfected with the vector alone) were also isolated.

Western blotting

For the direct detection of expressed proteins, cells were extracted with the lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 0.1% SDS). 50 μg protein from the cell lysate was electrophoresed in a 10% SDS-polyacrylamide gel. After electrophoresis, protein bands were electrophoretically transferred onto nitrocellulose membranes. The proteins on the membrane sheets were incubated with antibodies against annexin 6 (rabbit polyclonal or mouse monoclonal) and annexin 4 (mouse monoclonal). After the incubation with HRP-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG, detection was performed using ECL western blotting detection reagents (Amersham Pharmacia Biotech, USA). The blotting of the deletion mutants of annexin 6 was performed with anti-hemagglutinin-A rabbit polyclonal antibody.

Flow cytometry analysis

Both the annexin 6 stably expressing transfectant, Anx#1, and the mock vector transfectant, mock#1, were cultured in DMEM supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin and 0.2 mg/ml Geneticin (Sigma). Cells cultured up to subconfluency were rinsed twice with PBS (–) and harvested by treatment with 0.05% trypsin in PBS (–) at 37°C for 3 minutes. Cells were collected, washed twice with 1 mg/ml trypsin inhibitor in PBS (–) and resuspended in the growth medium at 37°C for 10 minutes to allow cells to recover. Cells (1×10^6) were suspended in 0.5 ml of DMEM containing 1% BSA (FACS buffer) in 1.5 ml microcentrifuge tubes and cooled to 4°C. The rabbit anti-annexin 6, rabbit anti-focal adhesion kinase (FAK), rabbit anti-β-1 integrin or various anti-annexin 4 antibodies were added to each tube. Cells were incubated with the primary antibody at 4°C for 1 hour, washed twice with FACS buffer and resuspended in the FACS buffer containing a secondary mouse FITC-conjugated anti-rabbit or anti-goat IgG (1:1000 dilution) and 5 μg/ml propidium iodide at 4°C for 30 minutes (Martin et al., 1995). Cells were then washed three times with FACS buffer, resuspended in 0.5 ml medium at 4°C and subjected to flow cytometric analysis using ABI model FACScan flow cytometer (Chung and Erickson, 1994; Tressler et al., 1994).

Results

Occurrence of proteins that bind to chondroitin sulfate chains on the cell membranes

We investigated the effect of CS chains on the first step of cell adhesion, cell attachment to substrata, using a modified cell attachment assay (Friedlander et al., 1988; Ernst et al., 1995). Fig. 1 shows the sequential steps in this assay. CS was conjugated to the lipid PE (producing CSPE), which has a hydrophobic domain. This lipid enabled us to quantitatively and reproducibly immobilize CS chains in plastic wells, and the CSPE substratum was used to investigate the effect of CS chains on the cell-substratum adhesion. In this assay, when FN-coated or ConA-coated substrata were used, human fibroblast IMR90 cells strongly attached to these substrata (Fig. 2A). Cell attachment to FN was greatly inhibited by 2 mM GRGDSP (RGD) peptide added to the medium but not by 2 mM GRGESP (RGE) peptide, whereas neither 2 mM GRGDSP nor 2 mM GRDESP affected cell attachment to ConA (Fig. 2A). The results indicated that, in this assay, the attachment of the cell to FN substratum was dependent on the specific interaction

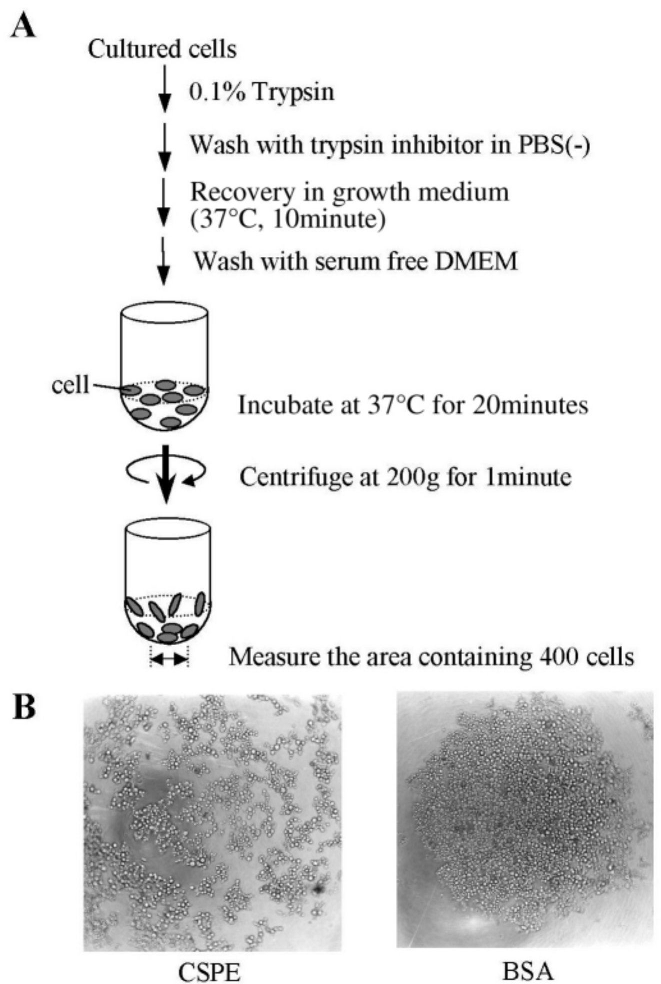

Fig. 1. A schematic representation of the cell attachment assay (A) and the attachment of cells to CSPE-coated substratum in this assay (B). When cells were plated onto adhesive- or 5 μg/ml CSPE-coated wells, they remained attached to the entire bottom of the well after the centrifugation. When cells were plated onto non-adhesive protein, BSA (10 mg/ml), the centrifugation formed a small pellet of cells at the bottom of the well.

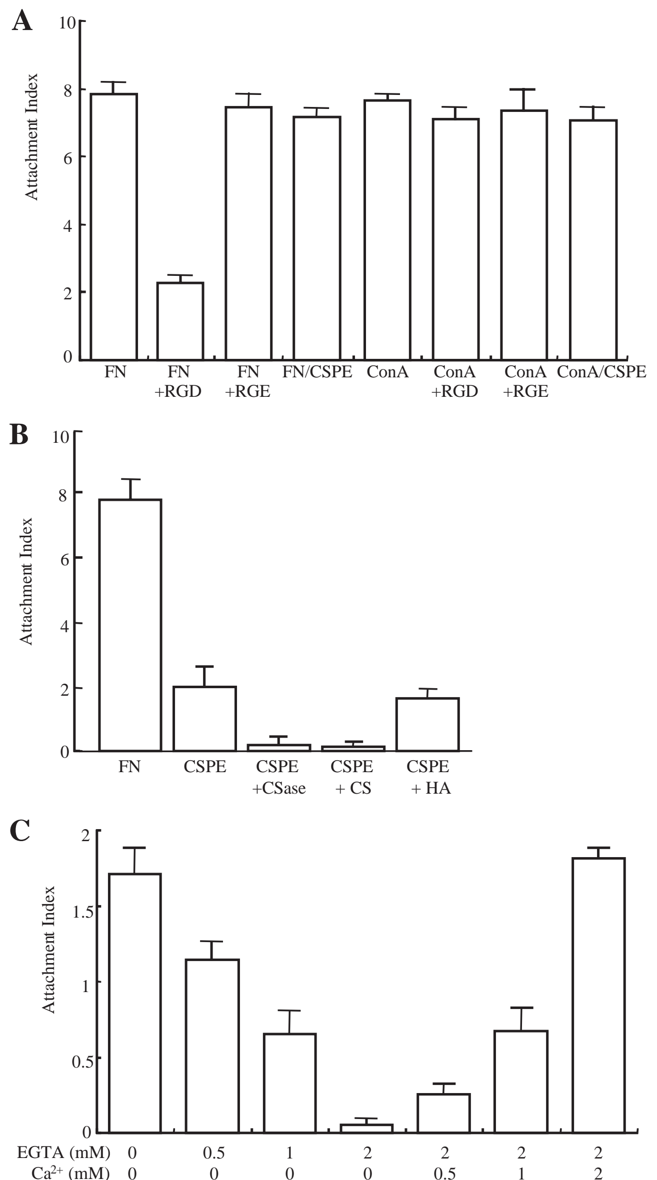

Fig. 2. (A) Effect of the simultaneous presence of CS chains in the substratum on cell attachment to FN or ConA substratum. The attachment of human IMR90 cells was assessed by the cell attachment assay as described in the Materials and Methods. The wells were coated with 5 μg/ml FN, 8 μg/ml ConA and 5 μg/ml CSPE. +RGD, the addition of 2 mM GRGDSP peptide to medium; +RGE, the addition of 2 mM GRGESP peptide to the medium. (B) Attachment of cells to CSPE substratum via a specific interaction with CS chains. Compared with the attachment to FN, human IMR90 cells weakly but significantly attached to CSPE substratum. +CSase, the pretreatment of the wells with chondroitinase ABC; +CS, the addition of 1 mg/ml CS (shark cartilage) to the medium; +HA, the addition of 1 mg/ml HA to the medium. The addition of CS (whale cartilage) and dermatan sulfate at the same concentration as above had the same inhibitory effect, and chondroitin had no significant effect (data not shown). (C) Dependency of the cell attachment to CSPE substratum on Ca^{2+}. IMR 90 cells were plated onto CSPE-coated wells containing the media with variable concentrations of EGTA and Ca^{2+}. The results are expressed as mean values and standard deviations of determinations from at least three independent experiments.

of integrin with FN. Thus, it is likely that the observed cell attachment to substrata reflects the interactions of cell surface receptors and substrata. When substrata were doubly coated with FN and CSPE, or ConA and CSPE, IMR90 cells attached to those molecules at the concentration of 5 μg/ml CSPE (Fig. 2A). We had previously reported that CSPE substratum at this concentration completely inhibited cell spreading to FN or other adhesive substratum (Sugiura et al., 1993). The above result suggests that CS chains had no inhibitory effect on cell-substratum interactions mediated by cell surface receptors such as integrin, at least under the assay conditions used here. Taken together, the results suggest that the inhibitory activity of CS chains to cell adhesion was not caused by steric hindrance of the interactions between cell surface receptors and adhesive protein substrata and that CS chains do not inhibit cell attachment to adhesive protein substrata but inhibit cell spreading on those substrata.

IMR90 cells weakly but significantly attached to CSPE substratum (Fig. 2B). When CSPE substratum was treated with

chondroitinase ABC, IMR90 cells were not able to attach to this substratum (Fig. 2B), indicating that CS chains are essential for the attachment and that the PE portion is not implicated in the cell-CSPE substratum interaction. These results suggest that the attachment of cells to CSPE substrata is caused by the direct interactions between cells and CS chains. Further, the attachment of cells to the CSPE substrata was altered by the addition of free CS chains (from shark cartilage and whale cartilage) to the assay medium, but not by addition of hyaluronan or chondroitin (Fig. 2B). Dermatan sulfate produced the same interfering effect at almost the same concentration, but heparin caused cell-cell aggregation at even lower concentrations (data not shown). Thus, alteration of cell-attachment to CSPE substratum seemed to be dependent on the concentration of disengaged CS (data not shown). It is likely, therefore, that the attachment of cells to CSPE substratum is not caused by direct and specific interactions between cells and CS chains; instead, it suggests that there is a receptor for CS chains on the cell surface.

The addition of EGTA to the assay medium containing 1.8 mM Ca^{2+} decreased the attachment of cells to the CSPE substratum, and the further addition of Ca^{2+} recovered the attachment in a stoichiometric manner (Fig. 2C), indicating the involvement of Ca^{2+} in the attachment of cells to CSPE substratum.

Purification and characterization of chondroitin-sulfate-binding proteins

We then embarked on the characterization of molecules that bind to CS chains in a Ca^{2+}-dependent manner on the cell surface. We prepared the membrane extract from cultured human fibroblast WI38 cells. The extract was then subjected to the CS affinity column, and the bound proteins were eluted as described in the Materials and Methods. The eluted fraction was concentrated and subjected to SDS-PAGE in 10% polyacrylamide gels. The proteins were transferred onto a PVDF membrane and visualized by Coomassie brilliant blue staining (Fig. 3A). The two protein bands of 68 kDa and 35 kDa were detected in the affinity fraction (the elution buffer). CS affinity column chromatography using the membrane extract of cultured chick embryonic fibroblasts revealed only the 68 kDa band in the affinity fraction (Fig. 3A). The 68 kDa band from human fibroblast WI38 cells was analyzed for the amino-acid sequence. Direct sequencing was unsuccessful, probably because of an N-terminal modification. Three internal sequences, ELKWGTDXAQFI, LSAXARVXLK and EDXQVQAA, were obtained from the digests of the 68 kDa band with V8 protease. These partial sequences correspond to human annexin 6 (Fig. 3B). In addition, the human 68 kDa band was specifically recognized using anti-annexin antibodies (Fig. 3C). We also identified the 68 kDa protein from chick fibroblasts by amino-acid sequencing, and we found it to be the partial sequence of chicken annexin 6 (Fig. 3B) (Cao et al., 1993). We concluded that the 68 kDa bands in both the affinity fractions were annexin 6 and suggested that it might be a candidate receptor for binding to CS chains on the cell surface. We also characterized the 35 kDa protein band in the human sample. Sequence analyses of peptide fragments following V8 protease digestion showed that they were human annexin 4

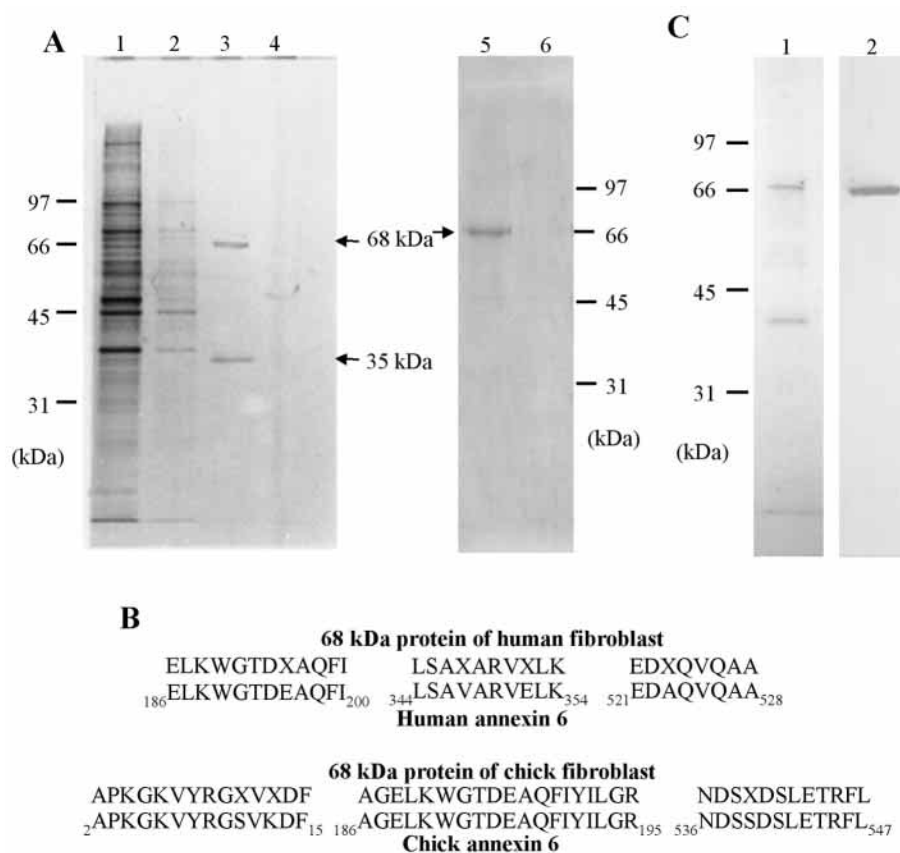

Fig. 3. Purification and characterization of a putative receptor for CS chains. (A) Human fibroblast WI38 extracts (lanes 1-4) and chick embryonic fibroblasts (lanes 5 and 6) were subjected to CS affinity chromatography in the presence of Ca^{2+}. Cell membrane extracts (lane 1), the flow-through fraction from the CS column (lane 2), the elution fraction with EDTA buffer (lanes 3 and 5) and the subsequent fraction obtained by further elution with 4M urea (lanes 4 and 6) were subjected to SDS-PAGE (10%) and visualized by Coomassie brilliant blue staining. (B) Comparison of the amino-acid sequence of the 68 kDa protein with annexin 6. (C) Proteins in the elution fraction from human WI38 fibroblasts extracts were subjected to SDS-PAGE and western blot analysis. Lane 1, Coomassie brilliant blue staining of SDS-PAGE (10%). Lane 2, identification of immunoreactive human annexin 6 in the elution fraction by western blot analysis with rabbit anti-annexin 6 antibodies. An identical blotting pattern was obtained by analysis using mouse monoclonal antibody to human annexin 6.

fragments (data not shown). These results suggest that annexin 4, as well as annexin 6, is a receptor for CS chains in human cells. However, since annexin 6 was identified in both human and chicken cells, we focused on this molecule.

Possible role of annexin 6 as a receptor for chondroitin sulfate on the cell surfaces

We then examined whether annexin 6 acts as a receptor for CS chains on the cell surface using the cell attachment assay. A431 cells did not attach to the CSPE substratum, whereas IMR90, MG63, MRC5 and WI38 cells did (Fig. 4A). Interestingly, the A431 cell line is so far only the cell line known to lack endogenous annexin 6 (Theobald et al., 1994), and we confirmed this lack of expression by western blotting (Fig. 4B). Annexin 4 was expressed in all of the cell lines examined. The results suggest that annexin 6, but not annexin 4, participates in the binding of cells to the CSPE substratum. To confirm the function of annexin 6 in binding to CS chains, we expressed it in A431 and examined whether attachment of these cells to the CSPE substratum was recovered. We transfected the full-length human annexin 6 cDNA into A431 cells (Fig. 5A). Two A431 cell lines, Anx#1 and Anx#2, that were stably expressing annexin 6 were obtained by transfection of the full-length human annexin 6 cDNA. The transfectants attached to CSPE substrata, but the parent A431 cells and the mock transfectants did not (Fig. 5B). Moreover, the extent of the attachment to CSPE substratum was increased with higher levels of expression of annexin 6 (Fig. 5A,B). These results strongly suggest that specific attachment can occur on CSPE substratum via annexin 6.

In contrast to other annexins, which have a structural motif of four repeats in the central core region, annexin 6 has eight repeats (Raynal and Pollard, 1994; Edwards and Moss, 1995) (Fig. 6A). Analysis of the crystal structure of annexin 6 also indicates that it is uniquely organized into two lobes, the N-terminal half (from repeat one to four) and the C-terminal half (from repeat five to eight) of the molecule (Kawasaki et al., 1994; Benz et al., 1994), and each lobe has convex and concave sides and a hydrophilic pore surrounded by the four repeats that might be involved in GAG interactions. In vitro, the C-terminal half, prepared by digestion with V8 protease of bovine brain annexin 6, bound to chondroitin sulfate in a Ca^{2+}-dependent manner (Ishitsuka et al., 1996). Furthermore, exon 21 was alternatively spliced, giving rise to two annexin 6 isoforms that differ with respect to a six amino-acid insertion at the starting site of repeat seven (Smith et al., 1994).

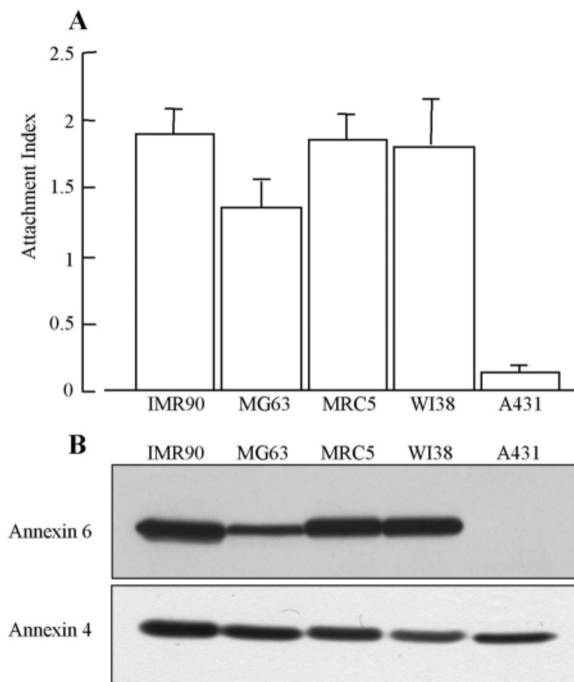

Fig. 4. The inability of A431 cell to attach to CSPE substratum is due to lack of annexin 6 expression. (A) The ability of various human cultured cell lines, IMR90, MG63, MRC5, WI38 and A431 cells to attach to CSPE substratum was examined using the cell attachment assay as described in the Materials and Methods. The results are expressed as the means and ranges of determinations from at least three experiments. (B) 50 μg of protein from cell lysates were analysed by western blotting of annexin 6 and annexin 4 with mouse monoclonal antibodies to human annexin 6 and human annexin 4, respectively.

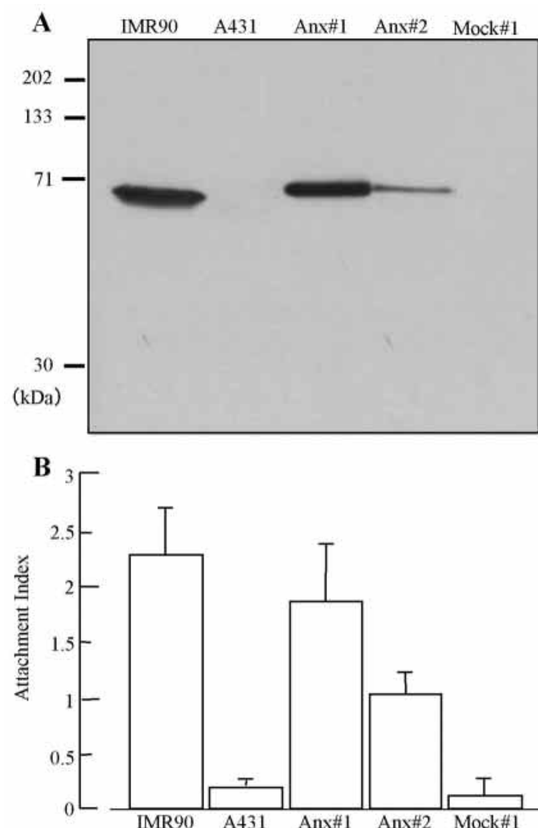

Fig. 5. The relationship between the expression of annexin 6 and the cell attachment ability of A431 cells. (A) The expression of annexin 6 was examined by western blotting of two stable transfectants with annexin 6 cDNA (Anx#1 and Anx#2) and one with mock vector (Mock#1), together with IMR90 and the parent A431 cells. 50 μg of protein of cell lysates were used for western blotting. Human annexin 6 was detected using mouse monoclonal anti-annexin 6 antibody. (B) Attachment of the transfectants to the CSPE substratum was assayed. The results are expressed as mean values and standard deviations of determinations from at least three independent experiments.

Considering these unique structures of annexin 6, we examined which region of annexin 6 is essential for the unique cell-CS binding and whether or not the alternatively spliced form has the same activity. We made five different deletion mutants of annexin 6 and examined the ability of cells with these mutants to attach to CS chains (Fig. 6A). The transfectant expressing the alternative splicing variant form of annexin 6 (ALT#5) attached to CSPE substrata as well as those expressing wild-type annexin 6 (WT#4) did. The transfectant expressing either the N-terminal or the C-terminal half (N#3, C+#2, C-#5) hardly attached to CSPE substratum (Fig. 6B). The results suggest that both lobes of annexin 6, the N- and C-terminal halves of the molecule, are necessary for specific attachment of the annexin-6-expressing cells to CS chains.

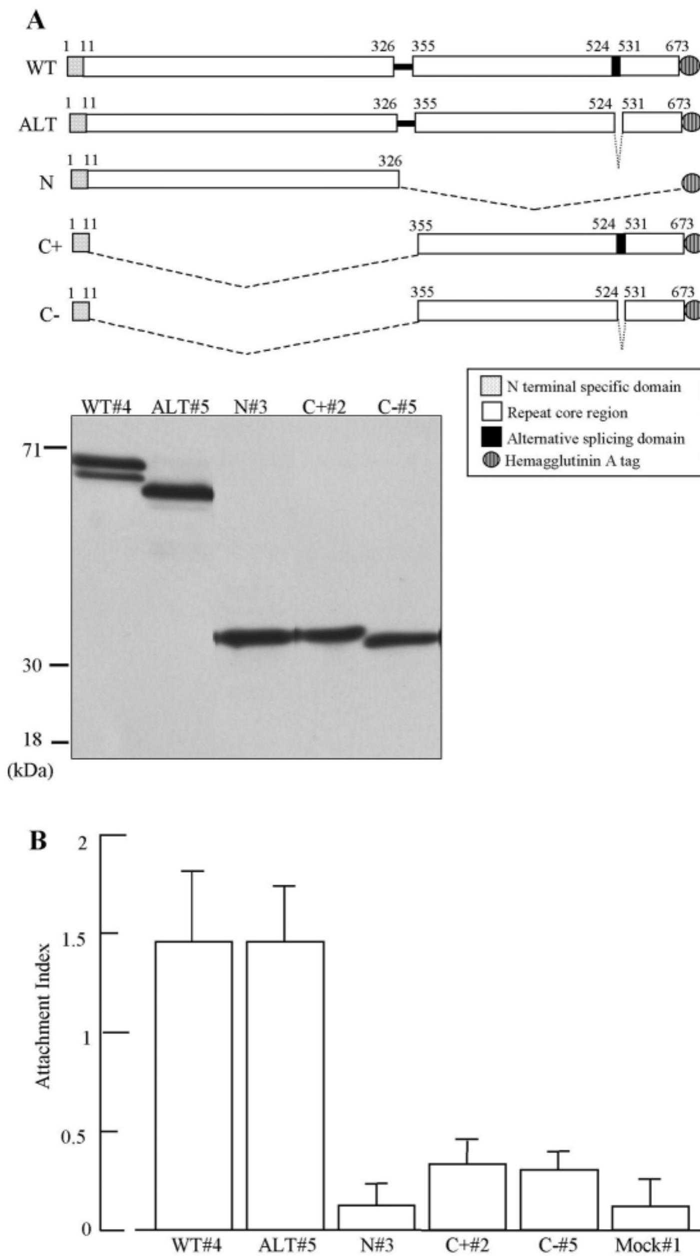

Fig. 6. Expression of the deletion mutants of annexin 6 and the effect on attachment to the CSPE substratum. (A) Schematic representation of the deletion mutant structures of human annexin 6. The abbreviations of the mutants used in the figure were indicated in the text. All constructs were designed with a hemagglutinin A tag at the C-terminus. The numbers indicate human annexin 6 amino-acid residues encoded by each construct. Western blotting of transfectants with those deletion mutants was performed using 50 μg of protein from cell lysates. The proteins were detected using anti-hemagglutinin A antibodies as described in the Materials and Methods. (B) The attachment abilities of those transfectants to CSPE substratum were examined using the cell attachment assay. The results are expressed as mean values and standard deviations of determinations from at least three independent experiments.

Occurrence of annexin 6 on cell surfaces

Annexin family proteins, including annexin 6, are cytoplasmic proteins. However, if annexin 6 functions as a receptor for CS chains, it must be exposed on the external cell surface membrane. Some reports have described the extracellular expression of annexins on the outer plasma membrane (Kirsch and Pfaffle, 1992; Yeatman et al., 1993; Chung and Erickson, 1994; Tressler et al., 1994). We, therefore, examined whether annexin 6 was actually located on the outer surface of cell membranes. We also measured the population of damaged or leaky cells using propidium iodide or FAK staining. By flow cytometry analysis, Anx#1 was positively stained with anti-annexin 6 polyclonal antibodies. Under the same condition, less than 5% of cells were stained with polyclonal antibodies to an intracellular antigen, focal adhesion kinase (FAK) or with propidum iodide (Fig. 7A). When the cells were incubated with anti-annexin 4 polyclonal antibodies, very few cells were stained. To avoid the possibility that the epitopes were not exposed to the antibodies, other antibodies to annexin 4 (two different monoclonal antibodies) were also used. Again, only a few cells were stained. These results indicate that a significant amount of annexin 6 is exposed on the external cell surface membrane. Thus, it is likely that annexin 6 functions as a receptor for CS chains and is involved in the anti-adhesive activity of CS proteoglycans.

Discussion

During cell-substratum adhesion, cells undergo attachment, spreading and form stress fibers and focal adhesion; these are sequential steps requiring different molecular mechanisms (Sage and Bornstein, 1991; Murphy-Ullrich, 1995; Murphy-Ullrich, 2001). CS proteoglycan PG-M/versican has an inhibitory effect on cell-substratum adhesion, and CS chains are needed for this activity, and the immobilization of CS chains on substrata is essential (Yamagata et al., 1989). In this study, we aimed to ascertain the underlying molecular mechanism of attachment. The candidate molecule purified by CS affinity chromatography in the presence of Ca^{2+} was analysed by peptide sequence analysis and reactivity with specific antibodies and shown to be annexin 6 (Fig. 3). Ca^{2+}-dependent binding of GAGs to annexins has been reported and characterized in several in vitro studies. Annexins 2 and 5 exhibit Ca^{2+}-dependent interactions with heparin and heparan sulfate chains (Kassam et al., 1997; Capila et al., 1999). The Ca^{2+}-dependent, carbohydrate-binding proteins from bovine kidney, which were purified by their Ca^{2+}-dependent affinity for CS chains, are annexin 4 and annexin 6 (Ishitsuka et al., 1998). In addition, we have examined the binding affinity of

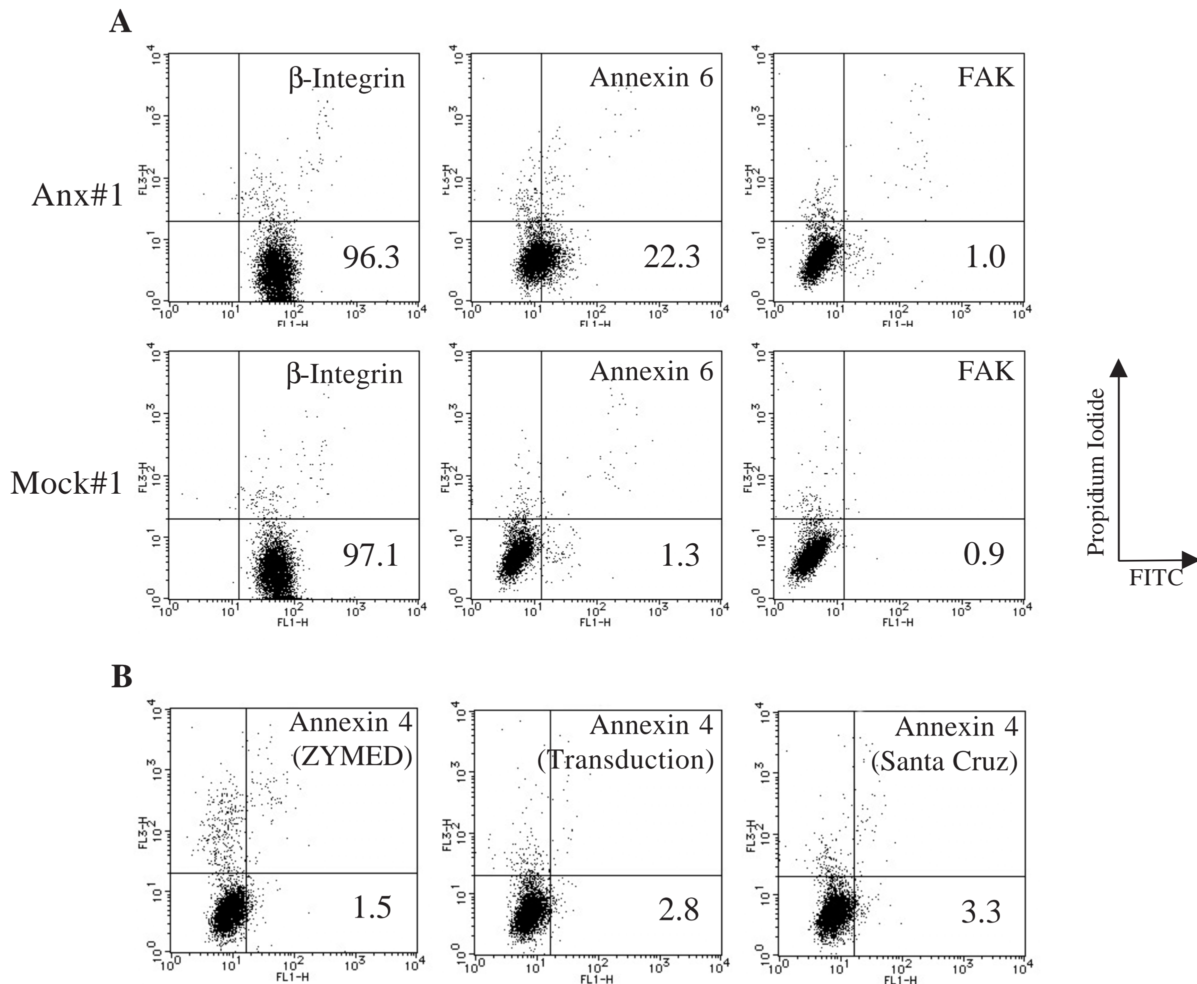

Fig. 7. Flow cytometric analysis of the annexin-6-expressing transfectant. (A) One million stable transfectants with the annexin 6 cDNA (Anx#1) or mock vector (Mock#1) were labeled with various antibodies. Anti-FAK antibody (1:200 dilution) was used as a negative control. Anti-annexin 6 and anti-β1-integrin polyclonal antibodies were used at the final dilutions of 1:500 and 1:200, respectively. (B) Anx#1 was also labeled with two different monoclonal (ZYMED and Transduction Laboratories) and one polyclonal antibodies (Santa Cruz) to annexin 4 at the final dilutions of 1:200. The source of the anti-annexin 4 antibody is indicated in brackets. Propidium iodide was used for the detection of any damaged or leaky cells. Gates for propidium iodide staining and FITC intensity were placed on the basis of values of negative cells. Numbers in quadrants represent the percentage of gated cells.

annexin 6 to CS by capillary zone electrophoresis according to the method of Honda et al. (Honda et al., 1992). The dissociation constant of the annexin-6-CS interaction was 9.4×10^{-5} M (data not shown). Taken together, it is reasonable to conclude that annexin 6 is a receptor for CS chains or that it, at least, binds to CS on the cell surface in the presence of Ca^{2+}.

Although annexin 6 is a cytoplasmic protein, we and another group have shown that annexin 6 also exists on the cell surface (Fig. 7) (Tressler et al., 1994). A number of recent papers have reported that cytoplasmic proteins can be secreted into the extracellular phase by an unknown mechanism; cytoplasmic proteins can also be found on the cell surface as receptors for various proteins. For example, annexin 5 was identified as a receptor for type II collagen, which used to be called anchorin CII (Kirsch and Pfaffle, 1992). Annexin 2 is a receptor for the alternatively spliced segment of FN type III domains in tenascin-C (Chung and Erickson, 1994) and for plasminogen and tissue plasminogen activator (Hajjar et al., 1994). In addition, calreticulin is a widely expressed Ca^{2+}-binding protein found mainly in the endoplasmic reticulum but also in other cellular compartments and could be functional as a receptor for thrombospondin (Goicoehea et al., 2000). These reports also support the present notion that annexin 6 exists on cell surfaces and is a receptor for CS.

In the present study, we have shown that human A431 cells devoid of annexin 6 expression were not able to attach to the CSPE substratum. A431 cells would, therefore, be insensitive to the inhibitory effect of CS proteoglycans or immobilized CS on cell-substratum adhesion if annexin 6 were the receptor. However, A431 cells could not adhere to and spread on FN-CSPE substratum (data not shown). We suggested that annexin 4 as well as annexin 6 might be a receptor for CS chains in human cells (Fig. 3). In addition, although annexin 4 has an affinity for CS, as observed using the in vitro system, its binding to CS is also Ca^{2+} dependent (Ishitsuka et al., 1998).

Therefore, the observed sensitivity of A431 cell to the inhibitory effect of CS-immobilized CS on cell-substratum adhesion might be explained by this redundancy.

The molecular mechanisms of anti-adhesive activities of other proteins have been characterized recently. For example, tenascin is an anti-adhesive substratum molecule, and the active region that induces focal adhesion disassembly has been identified as the alternatively spliced FN type III repeats of tenascin (TNfnA-D) (Murphy-Ullrich et al., 1991). TNfnA-D when added to confluent endothelial cells reduced the number of positive focal adhesions on the cell by 50%, and this activity was substantially blocked by an affinity-purified annexin 2 antibody (Chung et al., 1996). Moreover, the ability of TNfnA-D to stimulate loss of focal adhesions was blocked by Rp-8-Br-cGMPS and KT5823 at the concentrations that selectively inhibit cyclic GMP-dependent protein kinase. These results indicate that annexin 2 is a receptor for TNfnA-D and that cyclic GMP-dependent protein kinase mediates focal adhesion disassembly triggered by tenascin (Murphy-Ullrich et al., 1996). However, inhibitors of signaling, as far as we have tested with those that are commercially available, did not significantly effect the anti-adhesive activity of CS proteoglycans and immobilized CS chains (data not shown). These results suggest that the inhibitory mechanism for CS chains may be different from that for tenascin. However, the anti-adhesive mechanism of immobilized CS chains is hardly known. It is interesting to speculate how CS chains inhibit cell spreading. Some signaling molecules that interact with annexin 6 have been described. Recent reports indicate that the interaction of annexin 6 and protein kinase Cα is dependent on the presence of Ca^{2+} and phosphatidylserine (Schmitz-Peiffer et al., 1998). Additionally, annexin 6 makes a complex with $p120^{GAP}$ (Ras GTPase-activating protein), Fyn (Src family kinase) and Pyk2 (focal adhesion kinase family member). In addition, annexin 6 directly binds to $p120^{GAP}$ and Fyn (Chow et al., 2000). It may be possible that the interaction of CS chains and annexin 6 causes the state change of these signaling molecules and induces the anti-adhesion activity.

In most eukaryotic cell surfaces, there are specialized lipid microdomains, rafts, that are plasma-membrane assemblies enriched in cholesterol and glycosphingolipids and that are involved in cell signaling at the plasma membrane. A variety of signaling molecules – glycosyl-phosphatidylinositol-anchored protein, tyrosine kinases, GTP-binding proteins – are concentrated in rafts (Anderson, 1998). This localization increases the concentration and stability of signaling molecule complexes, with a direct enhancing effect on signaling levels (Kholodenko et al., 2000). Annexin 6 interacts with rafts at elevated intracellular Ca^{2+} concentrations (Babiychuk et al., 1999). Owing to the interaction of annexin 6 with CS chains on the cell surfaces, the membrane characteristics might be changed. This modification of membrane properties influences lipid microdomain organization and/or protein-membrane association, and as a result, might inhibit cell spreading. Our present findings raise many interesting questions about the mechanisms of the anti-adhesive activity of CS proteoglycans on cell-extracellular-matrix interactions.

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