Abstract. Chondroadherin (the 36-kD protein) is a leucine-rich, cartilage matrix protein known to mediate adhesion of isolated chondrocytes. In the present study we investigated cell surface proteins involved in the interaction of cells with chondroadherin in cell adhesion and by affinity purification. Adhesion of bovine articular chondrocytes to chondroadherin-coated dishes was dependent on Mg$^{2+}$ or Mn$^{2+}$ but not Ca$^{2+}$. Adhesion was partially inhibited by an antibody recognizing β1 integrin subunit. Chondroadherin-binding proteins from chondrocyte lysates were affinity purified on chondroadherin-Sepharose. The β1 integrin antibody immunoprecipitated two proteins with molecular mass ~110 and 140 kD (nonreduced) from the EDTA-eluted material. These results indicate that a β1 integrin on chondrocytes interacts with chondroadherin. To identify the α integrin subunit(s) involved in interaction of cells with the protein, we affinity purified chondroadherin-binding membrane proteins from human fibroblasts. Immunoprecipitation of the EDTA-eluted material from the affinity column identified α2β1 as a chondroadherin-binding integrin. These results are in agreement with cell adhesion experiments where antibodies against the integrin subunit α2 partially inhibited adhesion of human fibroblast and human chondrocytes to chondroadherin. Since α2β1 also is a receptor for collagen type II, we tested the ability of different antibodies against the α2 subunit to inhibit adhesion of T47D cells to collagen type II and chondroadherin. The results suggested that adhesion to collagen type II and chondroadherin involves similar or nearby sites on the α2β1 integrin. Although α2β1 is a receptor for both collagen type II and chondroadherin, only adhesion of cells to collagen type II was found to mediate spreading.

The cartilage extracellular matrix is highly specialized in its composition and organization to adapt to and withstand mechanical forces. A number of the matrix molecules are found predominantly or exclusively in cartilage (20). The major matrix components are collagens and proteoglycans (19), with collagen type II representing ~95% of the collagens (11) and aggrecan ~95% of the proteoglycans (16). Collagen type II fibers provide tensile strength to the tissue, whereas aggrecan, bound to hyaluronan, provides resilience. The interplay between these molecules is essential for cartilage function (33). Several other matrix components are involved in maintaining the specific cartilage properties, where some have primarily structural roles and others are associated with the chondrocytes and are likely to be involved in monitoring matrix properties and mediating signals to the cells (20). The chondrocytes, being the only type of cell in cartilage, have a key function in cartilage homeostasis. Their roles include controlling normal turnover of matrix molecules, depositing molecules into a functioning matrix, and responding to alterations in load with appropriate remodeling.

Chondroadherin (CHAD), originally described as a 36-kD protein, is a prominent noncollagenous extracellular protein in cartilage (31). Although the protein has been detected in extracts from cartilage and bone (31), recent data show very low expression of CHAD mRNA in bone while it is prominently expressed in certain zones of cartilage in young rats (Shen, Z., D. Heinegård, and Y. Sommarin, unpublished results). CHAD contains only a short oligosaccharide lacking sialic acid and hexosamines on serine 122 (31, 35). More recently its sequence was determined, both at the protein and cDNA level, showing that CHAD is a unique member of the leucine-rich repeat (LRR) protein family (35). Other members of this diverse family include the small cartilage proteoglycans biglycan (12), decorin (28), fibromodulin (36), lumican (2), and keratocan (6), as well as PRELP (1).

It has been shown earlier that isolated chondrocytes ad-
here to chondroadherin immobilized on plastic culture dishes (44) indicating that one function of this protein is to mediate interactions between the chondrocytes and the extracellular matrix. Fibroblasts and osteoblasts also adhered to CHAD (44), suggesting that a cell surface protein common to several cell types may be the receptor for the protein.

Integrins, a family of membrane glycoproteins, are of prime importance for adhesion of most cells to extracellular matrix proteins (22, 25, 37). They consist of two subunits, α and β, where the extracellular domain of the α subunits has several diverant, cation-binding sites. The integrins α1β1, α2β1, α3β1, α5β1, and α6β1 are common to several cell types and may be the receptor for the protein.

Materials and Methods

Antibodies

Monoclonal antibodies against the human integrin subunits β1 (P4C10), α2 (P1E6), α3 (P1B5), α5 (P1D6), and αv (VNR147) (unpurified ascites fluid) were from Life Technologies Inc. (Grand Island, NY). Monoclonal antibody against the human integrin β3 (RUU-PLF12, purified IgG) were purchased from Becton Dickinson (Bedford, MA). Monoclonal antibodies against the human integrins αvβ5 (P1F6) and αvβ3 (LM609) (purified IgG) were from Chemicon International, Inc. (Temecula, CA). The monoclonal antibodies against the human integrin subunits α1 (TS27; hybridioma supernatant) and α2 (PIH5; hybridoma supernatant) and rabbit polyclonal antibodies against rat β1 integrin were kind gifts from Drs. William Carter, (Fred Hutchinson Cancer Research Center, Seattle, WA; 3), Timothy Springer (Boston Blood Center, Boston, MA; 23), and Krister Rubin (Uppsala University, Uppsala, Sweden; 15), respectively. The monoclonal antibodies Gi9, Gi14, Gi19, and Gi26 (hybridoma supernatant) and rabbit polyclonal antibodies on adhesion was investigated, the cells were suspended in PBS (+Ca and Mg) and incubated with antibodies for 20 min at room temperature before plating of the cells. The monoclonal antibodies α1, α2 (P1E6), α3, α5, αv, and αvβ5 (unpurified ases fluid) were diluted 1:100, PIH5 (hybridioma supernatant) was diluted 1:25, and Gi9, Gi14, Gi19, and Gi26 (hybridioma supernatant) were diluted 1:10. The monoclonal antibodies β3 and αvβ3 were used at a concentration of 10 μg/ml. After 1 h of incubation the wells were gently rinsed with PBS to remove nonadherent cells. Adhesion was determined by measuring lysosomal hexosaminidase as described by Landegren (29).

Cell Isolation and Culture

Bovine chondrocytes were isolated by pronase digestion (Calbiochem, La Jolla, CA) digestion for 1 h followed by collagenase (CLS1; Worthington Biochemical Corp., Lakewood, NJ) digestion of articular cartilage from bovine chondrocytes or human lung carcinoma fibroblasts A549 were suspended in 1 ml of PBS containing 1 mg/ml glucose. 125I (1 mCi; Nordion Inc., Kanata, ON, Canada) was added to the cells together with 4 U of catalase (Sigma Chemical Co.; 120 U/mg) and 0.05 U of glucose oxidase (Sigma Chemical Co.; 1010 U/ml) prepared fresh in PBS-glucose. The cells were kept on ice for 15 min, whereafter the reaction was stopped by adding 10 ml of Dulbecco's culture medium. The cells were then washed three times with PBS and lysed for 1 h on ice in 2 ml of 1% Triton X-100, 100 μg/ml aprotinin, 2 μg/ml leupetin, 2 μg/ml pepstatin, 1 mM PMSF (Sigma Chemical Co.), 1 mM MnCl2, 1 mM MgCl2 in 10 mM Tris-HCl, pH 7.4. Cell lysates were centrifuged at 10,000 rpm for 30 min at 4°C, and the pellets were discarded.

Isolation and Coupling of CHAD to Agarose

CHAD was purified from bovine tracheal cartilage essentially according to the published procedure (31). For coupling, CHAD (2.5 mg) was solubilized in 0.5% SDS and coupled to 2 ml of Mini-Leak agarose (Biorad Chemicals, Lund, Sweden) according to the manufacturer's instructions. The control agarose was treated in a similar manner but in the absence of protein.

Affinity Purification of CHAD-binding Protein

The CHAD agarose (0.5 ml) and the control agarose (0.5 ml) were packed in mini-columns (Bio Rad, Hercules, CA) and equilibrated with at least 20 vol. of 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 1 mM MnCl2, and 1 mM MgCl2. The lysates from the 125I-labeled cells were passed over the control agarose two times and then incubated with the CHAD agarose in the mini-columns for 2–3 h with continuous end-over-end mixing. The CHAD agarose was washed with 15 vol of the equilibration buffer containing 75 mM NaCl, and the column was then eluted with 20 mM EDTA, 1 mM PMSF, 10 mM Tris-HCl, pH 7.4. The eluted protein peak was passed over
a desalting column (PD-10; Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 50 mM Tris, pH 7.4, 0.3 M NaCl, 1% Triton X-100, 0.1% BSA, 1 mM CaCl₂, 1 mM MgCl₂, and 1 mM PMSF. Samples of the affinity-purified proteins were then either precipitated by methanol/chloroform (48) or immunoprecipitated by antibodies followed by separation on 4–12% SDS-PAGE and visualized by autoradiography or image analysis using the BioImaging Analyzer Bas2000 (Fuji Photo Film Co., Tokyo, Japan).

Immunoprecipitation

Radiolabeled proteins were immunoprecipitated from cell lysate and from affinity-purified material. In experiments where lysates were immunoprecipitated they were passed over a desalting column (PD-10; Pharmacia Fine Chemicals) equilibrated and eluted with 50 mM Tris, pH 7.4, 0.3 M NaCl, 1% Triton X-100, 0.1% BSA, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM PMSF. Samples of the affinity-purified proteins were then either precipitated by methanol/chloroform (48) or immunoprecipitated by antibodies followed by separation on 4–12% SDS-PAGE and visualized by autoradiography or image analysis using the BioImaging Analyzer Bas2000 (Fuji Photo Film Co., Tokyo, Japan).

Statistics

Results are presented as means ± SD. Student’s t test was used to determine statistical significance.

Results

Adhesion of Cells to Chondroadherin

CHAD, immobilized on culture dishes, mediated adhesion of cells in a dose-dependent manner (Fig. 1). Maximal adhesion was seen at a coating concentration of 1.2 μg/ml. Adhesion of bovine chondrocytes to CHAD was dependent on divalent cations such that Mg²⁺ or Mn²⁺ but not Ca²⁺ was required (Fig. 2). Only a low number of cells adhered to the control BSA (Fig. 2). The adhesion to CHAD decreased by 2/3 in the presence of 100 μg/ml of a polyclonal rat β₁ integrin antibody compared to adhesion in the absence of antibody (Fig. 3). This indicated that β₁ integrins are involved in the adhesion of chondrocytes to CHAD. The control antibody had only a minor effect on the adhesion.

Statistics

Results are presented as means ± SD. Student’s t test was used to determine statistical significance.
Affinity Chromatography of CHAD-binding Cell Surface Proteins

To identify integrins with affinity for CHAD, Triton X-100-solubilized, 125I-labeled cell surface proteins from bovine chondrocytes were affinity purified on CHAD coupled to agarose. As shown in Fig. 4, two proteins with molecular weight ~110 and 140 kD (nonreduced) were eluted from the CHAD column with EDTA. These proteins were immunoprecipitated with a polyclonal antibody against β1 integrin. As shown in the figure, this β1 integrin showed two bands migrating corresponding to 120 (β1 chain) and 150 kD (α chain) upon SDS-PAGE under reducing conditions. In addition, a protein band with mobility corresponding to 100 kD was found, which may represent a degradation product of the β1 integrin. We were not able to further identify the α chain from the chondrocyte integrin, since available antibodies against human integrins showed too low cross-reactivity to bovine integrins. To identify the β1-associated α chain with affinity for CHAD, Triton X-100-solubilized, 125I-labeled cell surface proteins from human fibroblasts were affinity purified on the CHAD column. Proteins eluted from the CHAD affinity purification experiments were immunoprecipitated with monoclonal antibodies against the human integrin subunits β1, α1, α2, α5, and αv. Fig. 5 shows that the antibodies against the integrin subunits β1 and α2 immunoprecipitated an integrin dimer of similar appearance, while antibodies against α1, α5, and αv did not specifically immunoprecipitate integrins from the EDTA eluate. In a control experiment (Fig. 6) it was shown that these cells express a number of different integrins. Taken together, these results strongly indicate that the integrin α2β1 is a receptor for CHAD.

Inhibition of Cell Adhesion to CHAD by Integrin Antibodies

Fibroblasts were adhered to CHAD in cell adhesion experiments in the presence of antibodies against various integrin subunits. As shown in Fig. 7 antibodies against the α2 or β1 integrin subunits inhibited the cell adhesion to >50% while antibodies against β3, α5, αv, or αβ3, or αβ5 had no or only a minor effect on the adhesion. In contrast to the other antibodies, the α3 antibody stimulated the adhesion of fibroblasts to CHAD. In agreement with the affinity purification experiments, these results show that α2β1 is a CHAD-binding integrin.

To study whether α2β1 is involved in the adhesion of chondrocytes to CHAD, human chondrocytes were adhered to immobilized CHAD in the absence or in the presence of an antibody against the integrin subunit α2. The antibody partially inhibited the adhesion of chondrocytes in a dose-dependent manner (Fig. 8). Around 30% of the adhesion was inhibited at the highest antibody concentration. This result confirmed that α2β1 is a CHAD-binding integrin on chondrocytes.

Since α2β1 is a receptor for both collagen type II (24) and CHAD, we investigated the interaction of T47D-cells (cells that express the α2 but not the α1 subunit) with these two substrates, using various antibodies to the α2 integrin subunit (Fig. 9). The α2 antibodies inhibited cell adhesion to collagen type II and CHAD in a similar manner, although they were somewhat less effective in the CHAD experiment. Higher concentrations of the antibodies did
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Figure 6. Immunoprecipitation of integrins from human fibroblasts. 125I-labeled A549 fibroblasts were lysed with 1% Triton X-100, 100 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin A, 1 mM PMSF, 1 mM MnCl2, and 1 mM MgCl2 in 10 mM Tris-HCl, pH 7.4. Aliquots of the lysate were immunoprecipitated with monoclonal antibodies against the integrin subunits β1 (P4C10), β3 (RUU-PLF12), α1 (TS2/7), α2 (P1E6), α3 (P1B5), α5 (P1D6), αv (VNR147), and αvβ5 (P1F6). The immunoprecipitated proteins were separated by SDS-PAGE (4–12%) under nonreducing conditions and visualized by autoradiography.

Figure 7. Inhibition of fibroblast adhesion to CHAD by integrin antibodies. Culture dishes (48 well) were coated with CHAD (5 µg/ml) and blocked for nonspecific binding with BSA (0.25%). Human A549 fibroblasts were allowed to adhere to the dishes for 1 h at 37°C in the presence of monoclonal antibodies against the human integrin subunits β1 (P4C10), β3 (RUU-PLF12), α2 (G9), α3 (P1B5), α5 (P1D6), αv (VNR147), αvβ3 (LM609), and αvβ5 (P1F6). Nonadherent cells were removed by washing, and adhesion was determined by analyzing lysosomal hexosaminidase. The adhesion is expressed as a percentage of the control, and the numbers represent the mean of duplicate adhesion from three individual experiments ±SD. *P < 0.05; **P < 0.01; ***P < 0.001; Pβ1 = 0.002; Pα2 = 0.01; Pα3 = 0.02.

Figure 8. Inhibition of human chondrocyte adhesion to CHAD by α2 integrin antibodies. Culture dishes (48 well) were coated with CHAD (5 µg/ml) and blocked for nonspecific binding with BSA (0.25%). Human A549 fibroblasts were allowed to adhere to the dishes for 1 h at 37°C in the presence of various concentrations of the monoclonal antibody against the human integrin subunit α2 (G9). Nonadherent cells were removed by washing, and adhesion was determined by analyzing lysosomal hexosaminidase. The adhesion is expressed as a percentage of the control, and the numbers represent the mean adhesion ±SD from three wells in one of two experiments.
Figure 9. Inhibition of adhesion of T47D cells to CHAD (a) and to collagen type II (CII; b) by various α2 antibodies. Culture dishes (48 wells) were coated with 5 μg/ml of CHAD or CII and blocked for nonspecific binding with BSA (0.25%). T47D cells were allowed to adhere to the dishes for 1 h at 37°C in the absence or in the presence of monoclonal antibodies against the integrin subunits β1 (P4C10), β3 (RUU-PLF12), or various α2 antibodies. Nonadherent cells were removed by washing, and adhesion was determined by analyzing lysosomal hexosaminidase. The adhesion is expressed as a percentage of the control, and the numbers represent the mean of duplicate adhesion from three individual experiments ±SD. *P < 0.05; **P < 0.01; (a) Pβ1 = 0.004; PG9 = 0.006; PG19 = 0.026. (b) Pβ1 = 0.000; PPIE6 = 0.001; PPHS5 = 0.001; PG9 = 0.000; PG126 = 0.047.

Figure 10. Spreading of T47D cells on collagen type II (CII) or CHAD. Chamber slides (eight chambers) were coated with 5 μg/ml of CII (A and B) or CHAD (C and D) and blocked for nonspecific binding with BSA (0.25%). Human T47D cells (20,000/well) were plated onto the chambers and allowed to adhere and spread for 3 h at 37°C in the absence (A and C) or in the presence (B and D) of 10⁻⁸ M PMA. Nonadherent cells were removed by washing, and the adherent cells were fixed with 2% paraformaldehyde in PBS and stained with Meyer's hematoxilin and erythrosin. Spreading was visualized by light microscopy, and mean cell area (Table I) was calculated by image analyses using the Zeiss software KS400/V2.00.
regulation of integrin activity by divalent cations. The activity of several integrins, including α2β1, is stimulated by Mg2+ or Mn2+ and inhibited by Ca2+ (14).

Available antibodies did not immunoprecipitate the β1-associated α integrin subunit from bovine chondrocytes that mediated the adhesion to CHAD. The most likely explanation for this is that antibodies raised against human integrin subunits show weak or no cross-reactivity to many of the bovine chondrocyte integrins. From the molecular weight of the CHAD-binding α integrin subunit (140 kD nonreduced and 150 kD reduced), the fact that the apparent size increased upon reduction and that the adhesion was Mg2+ dependent, it is likely that the α subunit involved is α2. Since we know from FACs® analysis that isolated human primary chondrocytes from articular cartilage have relatively small amounts of the α2β1 integrin (Holmvall, K., L. Camper, and E. Lundgren-Akerlund, unpublished results), we chose to investigate CHAD-binding integrins on human fibroblasts. These cells express α2β1 as well as other integrins (Fig. 6). In affinity purification experiments we were able to show that the integrin α2β1 indeed is a CHAD-binding integrin. Antibodies against α5 and αv immunoprecipitated orders of magnitude—lower amounts of their respective integrins from the CHAD-agarose eluate. We further found that monoclonal antibodies against the subunits β1 or α2 inhibited the adhesion of cells to culture dishes coated with CHAD, while antibodies against the other integrin subunits had minor or no effect on the adhesion. In contrast to the lack of effects of the other integrin antibodies, the α3-integrin antibody appeared to stimulate the adhesion to CHAD. The findings corroborated further that the integrin α2β1 mediates the interaction between cells and CHAD. To confirm a participation of α2β1 integrins also in chondrocyte adhesion, we studied the adhesion of human chondrocytes to CHAD in the presence of the α2 antibody Gi9. We found that the antibody partially inhibited adhesion of cultured chondrocytes to CHAD (Fig. 8), which confirms that the integrin α2β1 indeed is involved in adhesion of chondrocytes to this substrate. In agreement with the fibroblast experiment the Gi9 antibody only partially inhibited the adhesion of human chondrocytes. This may indicate that another receptor in addition to α2β1 is involved in the adhesion of cells to CHAD. It is also possible that the immobilized CHAD mediate a high degree of nonspecific binding.

In previous experiments, it has been shown that adhesion of chondrocytes and chondrosarcoma cells to collagen type II was mediated by the integrins α1β1 and α2β1 (24). Since α1β1 is present on both chondrocytes (24) and fibroblasts (Fig. 5) and since this integrin appeared not to interact with CHAD in the affinity chromatography experiments (Figs. 4 and 5), it is unlikely that collagen contaminants in the CHAD preparation were mediating the cell binding. Since integrin α2β1 is also a receptor for collagen type II (24) we asked whether adhesion to collagen type II and to CHAD were mediated by similar mechanisms. One observation indicating that there is a difference in the α2β1 integrin binding to these ligands is that chondrocytes (37), T47D-cells (Fig. 10), and fibroblasts (data not shown) all spread on immobilized collagen type II, while adhesion to CHAD did not promote spreading. One explanation may be that different sites on the α2 chain are involved in adhesion to collagen type II and CHAD. To study this, we adhered T47D cells to the two substrates in the presence of various α2 antibodies. The monoclonal antibodies P1E6, PIHS, and Gi9 are known to block adhesion of cells to collagen. The monoclonal antibodies Gi19 and Gi29 have some inhibitory effect on adhesion of platelets to collagen, while Gi14 does not inhibit adhesion. (Santoso, S., personal communication) The T47D cells do not express collagen type II binding integrins other than α2β1 and were therefore particularly informative in these studies (45; Camper, L., and E. Lundgren-Akerlund, unpublished results). We found that the different α2 antibodies inhibited adhesion to collagen type II and CHAD in a similar manner, indicating that these ligands bind to similar or nearby sites on the α2β1 integrin (Fig. 9). Further experiments using α2 integrin antibodies recognizing other epitopes on the α2 subunit will be needed to elucidate the binding sites. Another explanation is that the binding of collagen type II and CHAD is regulated differently. It has previously been shown that α2β1 integrins from different cell types show different ligand specificity. α2β1 on platelets and melanoma cells bind collagen (27, 46), while α2β1 on other cell types binds both collagen and laminin (9, 30). Several factors including divalent cations, proteoglycans, and phospholipids have been suggested to modulate integrin activity, and it has been suggested that the degree of activation may regulate their ligand specificity (4). Since Mn2+ has been shown to increase the affinity between integrins and their ligands in affinity chromatography (13) and cell adhesion (10, 32), we tested the possibility that Mn2+ could stimulate cell spreading. However, Mn2+ appeared not to stimulate spreading of T47D cells on immobilized chondroadherin (data not shown). Phorbol esters such as PMA are known to mimic the effect of several different integrin-activating stimuli and to induce clustering of integrins (7, 42, 49). Protein kinase C may therefore be an important regulator of the integrin affinity and ligand specificity. Our finding that cells showed some spreading on CHAD in the presence but not in the absence of PMA (Fig. 10 and Table I) indicates that spreading of cells may require activation and altered affinity of the integrins. It also lends strong support to the involvement of integrins in the cell attachment.

It is likely that integrin α2β1, being a receptor for two different proteins in cartilage, has an important function in mediating signals between the chondrocytes and the cartilage matrix. We and others (8, 24, 50) have found that isolated chondrocytes express relatively small amounts of α2β1 integrin. The collagen type II binding integrin α1β1, on the other hand, is one of the major integrins on isolated chondrocytes. It is possible that α2β1 is a more dynamic integrin that is upregulated during changes in cell–matrix interactions such as matrix turnover, remodelling, or me-

### Table I. Spreading of T47D Cells on CII or CHAD in the Absence or in the Presence of PMA

|          | CII  | CHAD |
|----------|------|------|
| − PMA    | 123 ± 20 | 83 ± 8 |
| + PMA    | 208 ± 38 | 140 ± 10 |

The numbers represent mean cell area (μm²) of cells ± SD (n = 4).
also been shown that growth factors such as TGFβ (21) and EGF (5) stimulate expression of the integrin α2β1, indicating that growth factors can regulate the action of α2β1 in cartilage and to elucidate the differences between interaction of α2β1 with CHAD and collagen type II.

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