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Purification, crystallization and preliminary X-ray diffraction analysis of human chondroadherin

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Chondroadherin is a cartilage matrix protein that is known to mediate the adhesion of isolated chondrocytes. Its protein core is composed of 11 leucine-rich repeats flanked by cysteine-rich domains at the N- and C-terminal ends. Recombinant human chondroadherin was crystallized using the sitting-drop vapour-diffusion method. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 56.4$, $b = 111.3$, $c = 128.5$ Å, $\beta = 92.2$, and are most likely to contain four molecules in the asymmetric unit. The crystals diffracted to at least 2.3 Å using synchrotron radiation, but structure determination using molecular replacement has so far been unsuccessful.

1. Introduction

Chondroadherin is a noncollagenous extracellular matrix protein found in cartilaginous tissues, bone and tendon (Shen *et al.*, 1998). It was first isolated and purified from bovine cartilage (Larsson *et al.*, 1991) and its primary structure identified it as a member of the leucine-rich repeat (LRR) family (Neame *et al.*, 1994). Adhesion experiments have shown that articular chondrocytes can adhere to plastic culture dishes coated with chondroadherin (Sommarin *et al.*, 1989) in an Mg^{2+}- or Mn^{2+}-dependent manner (Camper *et al.*, 1997), which makes it likely that the function of the protein is to mediate the interaction of chondrocytes with the extracellular matrix. Chondroadherin has been shown to interact with integrin $\alpha_2\beta_1$ (Camper *et al.*, 1997) and it was therefore suggested that chondroadherin mediates signalling between cartilage matrix and chondrocytes. Fibroblasts and osteoblasts have also been observed to adhere to chondroadherin, which supports the idea that chondroadherin interacts with a cell-surface protein. Chondroadherin has also been shown to bind to collagen type II (Månsson *et al.*, 2001) and both collagen type II and chondroadherin interact with integrin $\alpha_2\beta_1$.

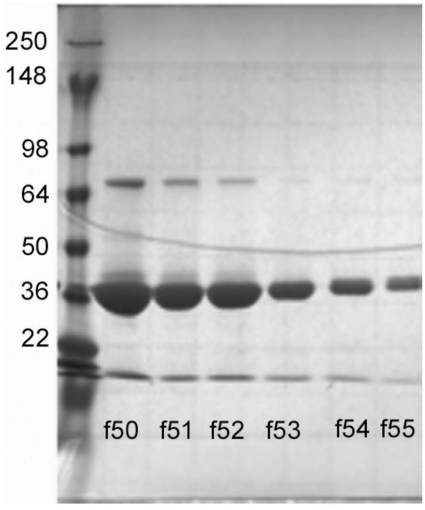
The dominant structural motif in chondroadherin is the leucine-rich repeat (LRR). The family of LRR proteins was identified by Patthy (1987) and subsequently refined by Kobe & Deisenhofer (1994). The repeats contain a consensus sequence in which leucines are conserved at fixed positions. The consensus sequence has the pattern *x*L*xx*L*x*L*xx*N±*xaxx*±*a*±±±±*a*±±*x*±±, where *x* is any amino acid, *a* is an aliphatic amino acid, L is leucine or isoleucine, ± indicates a looser requirement for a particular amino acid and N is generally a asparagine but can also be replaced by a cysteine or a threonine. The number of leucine-rich repeats can vary from one in platelet glycoprotein-Ib β (Roth, 1991) to 38 in the plant disease-resistance protein (Dixon *et al.*, 1996). The most common number of repeats in extracellular matrix proteins is 10–11 and chondroadherin contains 11. The average length of an individual LRR is 24 residues, which is the case for chondroadherin, but the length can vary from 20 to 29 amino acids, even within individual proteins. The first characterized protein containing LRRs was the serum leucine-rich glycoprotein (Takahashi *et al.*, 1985) and the first structure of a member of the LRR family was that of ribonuclease inhibitor (RI), which was solved to a resolution of 2.5 Å (Kobe & Deisenhofer,

1993). RI has a horseshoe-shaped overall structure with 15 LRRs. The structure has a typical pattern of β-strands on the inside of the horseshoe and helices on the outside. The subsequently characterized LRR-containing structures from the extracellular matrix, such as decorin (Scott *et al.*, 2004) and biglycan (Scott *et al.*, 2006), typically have a reduced curvature, which can be attributed to the fact that they have a lower helical content on the outside of the horseshoe (McEwan *et al.*, 2006).

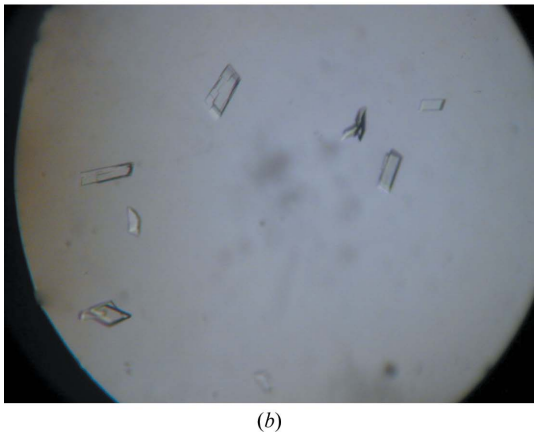
Chondroadherin belongs to the family of small leucine-rich repeat extracellular matrix proteins (SLRPs). The SLRPs possess disulfide clusters at both the N- and C-terminal ends of the LRR region. The SLRP family can be further divided into four subfamilies based on overall sequence similarity, the patterns of the cysteine residues and the gene structure (Hocking *et al.*, 1998; Iozzo & Murdoch, 1996). The subfamilies are class I (biglycan, decorin and asporin), class II (fibromodulin, lumican, keratocan, osteoadherin and PRELP), class III (epiphycan, osteoglycin and opticin) and class IV (chondroadherin). Chondroadherin is alone in its subgroup owing to differences in its primary structure and gene arrangement compared with other members of the family. It lacks an N-terminal extension, differs in its N-terminal cysteine-cluster pattern (Hocking *et al.*, 1998) and has only one putative poorly defined *O*-linked carbohydrate at Ser146 in bovine chondroadherin (Ser144 in human; Neame *et al.*, 1994), while classes I, II and III have putative sites for glycosaminoglycan (GAG) chains and *N*-linked oligosaccharides. Matsushima and coworkers have proposed that there are two distinct types of LRR in SLRPs, namely type S and type T (Matsushima *et al.*, 2000). The type S LRR is 21 residues long and has the consensus sequence *xxa*P*zx*L*p*-*xx*L*xx*L*x*L*xx*N*x*I. Type T has 26 residues and its consensus sequence is *zzxxaxxxx*F*xxaxx*L*xx*L*x*L*xx*N*x*L (where *x* indicates any residue, *z* is frequently a gap, *a* is Val, Leu or Ile and I is Ile or Leu). Chondroadherin is almost entirely composed of T repeats, whereas class I, II and III SLRPs all contain a mixture of S and T repeats.

Chondroadherin contains nine cysteine residues, of which Cys282–Cys324 and Cys284–Cys304 in the C-terminal domain (numbering as in the human sequence) have been identified as being involved in disulfide bridges (Neame *et al.*, 1994). Within the N-terminal domain, Cys23–Cys29 and Cys27–Cys38 have been found to make disulfide bridges (P. Önnerfjord, unpublished results). The ninth cysteine has been observed not to be involved in disulfide-bridge formation. It is located in the second LRR and is believed to be involved in stabilizing the structure.

Here, we describe the purification, crystallization and preliminary X-ray diffraction analysis of human chondroadherin. The structure of chondroadherin will provide clues to the mapping of interaction areas on the surface of the molecule. It will also reveal whether chondroadherin dimerizes using the inside of the horseshoe, as do decorin (Scott *et al.*, 2004) and biglycan (Scott *et al.*, 2006), or whether it is principally a monomeric protein. The crystal structure will also illuminate the role of the C-terminal domain, which differs from the other known structures within the superfamily. The structure will also help in the three-dimensional modelling of other SLRP-family members.



(*a*)



(*b*)

Figure 1
(*a*) SDS–PAGE gel showing the purity of the chondroadherin fractions eluted from a CM52 cation-exchange column. Fraction 53 was used for crystallization trials. Molecular-weight markers are labelled in kDa. (*b*) Crystals of chondroadherin grown under the condition 21% PEG 3350, 6% xylitol, 0.1 *M* glycine–NaOH pH 9.5 and 0.2 *M* ammonium sulfate. Typical dimensions are 0.15 × 0.1 × 0.05 mm.

2. Materials and methods

2.1. Expression and purification

Expression of recombinant human chondroadherin (UniProtKB/SwissProt accession No. O15335) in human kidney cells (293-EBNA, Invitrogen) was carried out as described previously (Månsson *et al.*, 2001). Full-length chondroadherin containing 359 amino acids was used.

90 ml cell-culture medium containing chondroadherin was centrifuged at 5000 rev min^{-1} for 5 min and the supernatant was filtered through 0.8 and 0.2 µm filters. The protein solution was diluted to 350 ml with Tris–HCl to give a final concentration of 20 m*M* at pH 7.0. A 20 ml CM52 cation-exchange column (Whatman) was equilibrated with four column volumes of 20 m*M* Tris pH 7.0 and the sample was added to the column. The proteins were eluted using a 200 ml gradient of 0–0.5 *M* NaCl in 20 m*M* Tris–HCl. 1.75 ml fractions were collected. Fraction 53, corresponding to a NaCl concentration of ∼200 m*M*, was used for crystallization experiments without further dialysis or concentration. The purity of the protein in the collected fractions as determined by SDS–PAGE is shown in Fig. 1(*a*). The gel shows a band slightly above 36 kDa. Since the expected molecular weight of chondroadherin is 38 260 Da, this demonstrates that the protein has no significant glycosylation.

2.2. Crystallization

To determine the initial protein concentration, the Pre-Crystallization Trial kit (Hampton Research, Aliso Viejo, California, USA) was used. Initial crystals grew within 1 d in solution A2. Initial crystallization trials were performed with the Crystallization Basic Kit for proteins (Sigma–Aldrich, Sweden) using the sitting-drop

Table 1
X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 56.4, b = 111.3, c = 128.5, β = 92.2
Wavelength (Å)	1.0000
Resolution range (Å)	14.9–2.3 (2.4–2.3)
$R_{\rm merge}$† (%)	8.6 (37.7)
Total observations	152964
Unique reflections	66924
Average redundancy	2.3 (1.7)
Completeness (%)	95.0 (94.3)
$I/\sigma(I)$	7.2 (2.0)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ are the i individual observations of each reflection hkl and $\langle I(hkl)\rangle$ is the value after weighted averaging.

vapour-diffusion method at 293 K. The drops were prepared by mixing 1 µl reservoir solution with 1 µl protein solution (1.2 mg ml^{-1} in 20 m*M* Tris pH 7.0 and 200 m*M* NaCl). The drops were equilibrated against 400 µl reservoir solution. The initial screen indicated that a PEG/ion screen should provide good crystallization conditions. We used the PEG/Ion Plus screen (Biogenova, Frederick, Maryland, USA). This screen was carried out in the same manner as the Crystallization Basic Kit. 13 out of 50 conditions gave testable crystals. Single crystals were produced in a condition containing 21% PEG 3350, 6% xylitol, 0.1 *M* glycine–NaOH pH 9.5–10.5 and 0.2 *M* ammonium sulfate at 293 K.

2.3. Data collection

The crystals providing the data presented here were soaked in a cryoprotectant containing 21% PEG 3350, 20% xylitol, 0.1 *M* CHES pH 9.5, 0.2 *M* ammonium sulfate and 10 m*M* K_2PtCl_4 for 10 min and flash-cooled in a nitrogen-gas stream at 100 K. Diffraction data were collected at 100 K on beamline I911-3 of MAX-lab, Lund using a 225 mm MAR Mosaic detector. 200 images with an oscillation range of 0.5° were collected. The data were integrated with the *XDS* package (Kabsch, 1993) and further processed using programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) run through the *CCP*4*i* interface (Potterton *et al.*, 2003). The data were intended to be from a heavy-atom derivative, but since no peaks could be seen in the isomorphous or anomalous difference Patterson maps and the data were collected to a significantly higher resolution than a previous native data set, the K_2PtCl_4 data set was used as native data. The crystals of chondroadherin were somewhat radiation-sensitive, possibly because of their high disulfide content.

3. Results

Typical crystals are approximately 0.15 × 0.1 × 0.05 mm in size and belong to space group $P2_1$, with unit-cell parameters a = 56.4, b = 111.3, c = 128.5 Å, β = 92.2° (Table 1). There are most likely to be four or five chondroadherin molecules in the asymmetric unit. Four molecules would give a solvent content of 53.8% and a probability of 0.41 (Kantardjieff & Rupp, 2003), while five molecules give a solvent content of 42.3% and a probability of 0.50.

Chondroadherin has limited sequence identity to portions of a wide range of LRR proteins at a level of around 25–30% for full-length chondroadherin. However, the regular nature of the LRR coupled with the relative anonymity of the overall structure of many LRR proteins and their partial internal symmetry make it difficult to find an optimal search model for molecular replacement. Apart from the N- and C-terminal extensions, the SLRPs lack distinguishing structural features (α-helices or characteristic globular domains) within the LRR region. The molecules appear to have a dimeric arrangement from the presence of a peak in the χ = 180° section of the self-rotation function at 76% of the height of the origin peak (Fig. 2*a*). However, all attempts to date to solve the structure by molecular replacement with a wide variety of search models and programs have been unsuccessful, most likely as a consequence of translational pseudosymmetry in the crystals. The native Patterson function shows two distinct peaks at (0.129, 0.473, 0.220) and (0.180, 0.500, 0.248) of approximately 20% of the height of the origin peak (Fig. 2*b*). The reason for the presence in the NCS of two pure translation peaks but only one strong twofold axis is currently unclear to us. It is possible that there are two noncrystallographic twofold axes with such similar orientations that they are indistinguishable in

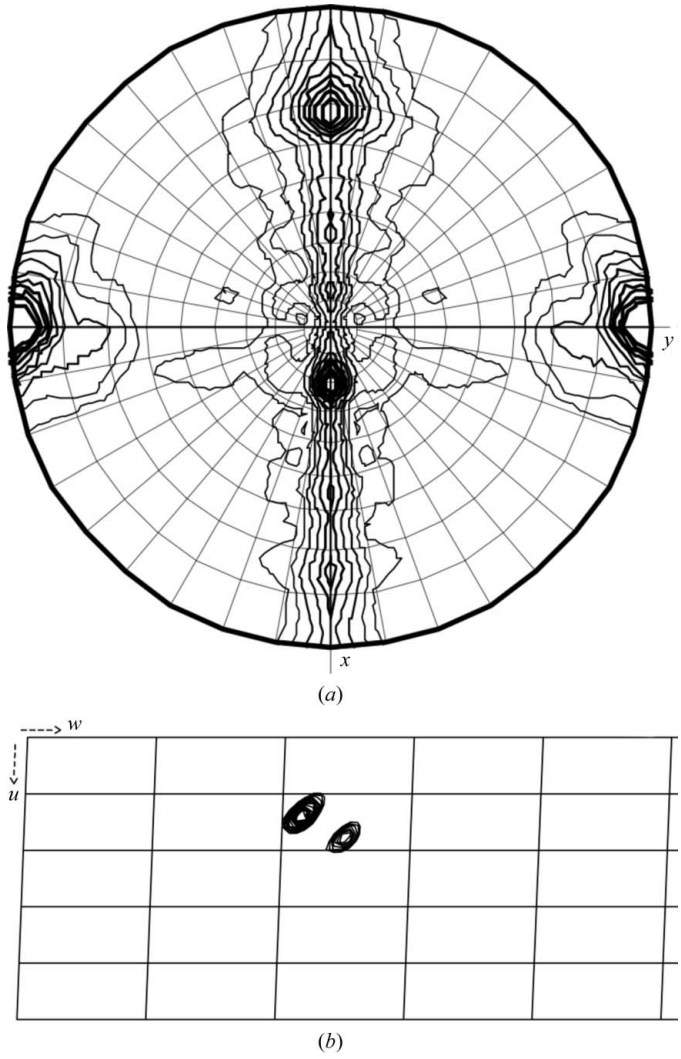


Figure 2
(*a*) Self-rotation function. The self-rotation function was calculated using *MOLREP* (Vagin & Teplyakov, 1997) and data from 20 to 2.3 Å resolution. The χ = 180° section is displayed with spherical polar angles and is contoured from 0.5 standard deviations (σ) above the mean in steps of 0.5σ. The strongest non-origin peak is at θ = 21.4°, φ = 0° with a height of 6.8σ (76% of the height of the origin peak). The second strong peak is at θ = 68.6°, φ = 180° and is related to the first by crystallographic symmetry. (*b*) Native Patterson function. The v = 0.5 Harker section is shown. The Patterson function was calculated using data from 15 to 2.3 Å resolution and is contoured from 3σ in steps of 0.5σ. A grid is drawn with a spacing corresponding to 0.1 of a unit-cell parameter. Two peaks with heights 20% and 19% of the origin peak can clearly be seen at u = 0.129, v = 0.473, w = 0.220 and u = 0.180, v = 0.500, w = 0.248. The reason for the existence of two peaks is currently unclear.

the self-rotation function at this resolution. Nevertheless, even when all this information is incorporated into the search process using *MOLREP* we cannot obtain molecular-replacement solutions that produce electron-density maps showing features that could lead to improvement of the model.

Heavy-atom soaks have been tested extensively, but so far no heavy atom has been found to bind. Different methods have been tested, such as the long-soak and quick-soak methods (Sun *et al.*, 2002) and halide soaks (Dauter *et al.*, 2000). Sulfur SAD (Dauter *et al.*, 1999) has also been tried; since chondroadherin has nine cysteines and three methionines in 359 residues, one w-ould expect a significant anomalous signal from sulfur, but the radiation-sensitivity of the crystals in combination with the low-symmetry space group has made it impossible to collect data of sufficient redundancy to solve the S-atom substructure. We are currently investigating the generation of better search models.

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