The Structure of Anchorin CII, a Collagen Binding Protein Isolated from Chondrocyte Membrane*

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cDNA clones for anchorin CII (M, = 34,000), a collagen-binding protein, were isolated from a Ygt 11 cDNA library prepared from chick cartilage mRNA. Several overlapping clones were characterized which gave rise to an open reading frame coding for 329 residues and a 3'-untranslated segment of 500 base pairs. The clones were identified as coding for anchorin by hybrid select translation analysis and by comparing the deduced amino acid sequence with the sequence of 10 tryptic peptides of the protein. A hydrophobic domain of 25 residues interrupted with 3 polar residues was identified with the carboxyl-terminal portion. There was no evidence for an amino-terminal signal peptide. Northern analysis revealed that the 5' probe hybridizes to a single 1.7-kilobases (kb) mRNA species, whereas the 3' probe hybridizes to two mRNA species of 1.7 kb and 5 kb, which are present in many cells including chondrocytes, crop cells, and fibroblasts. The level of anchorin mRNA in chick embryo fibroblasts was increased by infection with Rous sarcoma virus.

The interaction of cells with extracellular matrices influences their migration, adhesion, growth, and differentiation (for review, see Refs. 1–5). Such effects are probably specific and involve the binding of the cells to collagen, to proteoglycans, and to various glycoproteins such as fibronectin and laminin. Specific receptors for laminin and fibronectin have been described (for review, see Refs. 6 and 7), and recent studies suggest that there are also distinct cell surface receptors for collagens (3, 8–14). One of these is anchorin CII, a collagen binding protein first isolated from chondrocytes.

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The abbreviations used are: TEA, triethanolamine; Denhardt’s, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0; SDS, sodium dodecyl sulfate; bp, base pairs; kb, kilobases; PIPES, 1,4-piperazinediethanesulfonic acid.

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Anchorn CII cDNA

containing 0.1 mM CaCl₂ and 30 μg of trypsin at 37 °C for 14 h. The reaction was stopped by adding 1.5 ml of 0.1% trifuluoroacetic acid. The tryptic peptides eluted from the gel slices were separated on a Gilson high performance liquid chromatography gradient system. They were first separated on a Vydac RP18 reverse-phase column with a linear gradient (5-70%) in 0.1% trifluoroacetic acid. The resulting fractions were rechromatographed on a Merck Hilar reverse-phase column (LiChrospher CH18) with a more shallow acetonitrile gradient which was optimized for the purification of each peptide. Peptides isolated from the second reverse-phase column were concentrated in a Speed Vac concentrator. Between 100 and 1000 pmol of 13 tryptic peptides were sequenced using a spinning-cup sequinator (Beckman) or a gas-phase sequinator (Applied Biosystems).

Preparation of RNA and cDNA Synthesis—Total cellular RNA was extracted from 10-day-old chick embryos and from 16-day-old chick sternum, crop, calvaria, and fibroblasts using guanidine HCl extraction (21). Poly(A) RNA was isolated by chromatography on oligo(dT)-cellulose (22). Poly(A) RNA from 16-day-old chick embryo sternum was used as template for the synthesis of double-stranded cDNA. For this purpose, approximately 15 μg of poly(A) RNA was lyophilized and then incubated in the presence of 8 mM mthylmercuric hydroxide and 10 units of RNasin for 10 min at room temperature. After the addition of 90 mM 2-mercaptoethanol and 20 units more of RNase inhibitor, the mixture was further incubated for 15 min and then used as template for first strand synthesis. This reaction was carried out with 15 μg of oligo(dT) primer at 37 °C for 3 h in the presence of reverse transcriptase in 0.1 M Tris-HCl, pH 8.3, containing 0.14 M KC1, 0.01 M MgCl₂, and 0.8 mM concentration of each of unlabeled deoxyribonucleotide triphosphates; 10 μCi each of [α-32P]dGTP and [α-32P]dATP were used to quantitate cDNA synthesis.

Screening of the Chick cDNA Library—The chick chondrocyte cDNA library was screened by antibody to anchorin as described elsewhere (24, 25). Rabbit anti-anchorin antibodies (16) were used at 1:300 dilution for the detection of fusion proteins induced with isopropyl-1-thio-β-D-galactopyranoside in Escherichia coli Y1090 infected with the recombinant phage. Subsequently, filters were washed with TBS and incubated with 1:1000 horseradish peroxidase-conjugated goat anti-rabbit antibody for 2 h and developed with 4-chloro-1-naphthol (26). Positive plaques were identified, rescreened, and purified as described (27).

Characterization of Recombinant cDNA by Restriction Analysis and Southern Blotting—Restriction enzymes were used under the conditions recommended by the manufacturers. For Southern blotting, DNA was restricted, electrophoresed in agarose gels, transferred to nitrocellulose, and hybridized under standard conditions (28).

Northern Analysis—Either poly(A) RNA (1 μg) or total RNA (5 μg) was electrophoresed in 1% agarose gels with formaldehyde, transferred to nitrocellulose (29), and hybridized with nick-translated DNA probes at 37 °C in the presence of 5 × SSC, 50% formamide, 0.1% SDS, 250 μg/ml salmon sperm DNA, 5 × Denhardt’s solution, 50 mM Tris/HCl, pH 7.5, for 16-18 h. Filters were washed twice at 40 °C with 2 × SSC/0.2% SDS and twice with 2 × SSC. Labeled bands were detected by autoradiography.

DNA Sequencing— Sequencing of selected clones was performed in M13mp18 or M13mp19 using the dyeoxy method (30). The sequence of both strands was determined, and each nucleotide in sequence was identified some five times.

Hybrid Select Translation and Immunoprecipitation—Small circles (7 mm diameter) of ARM Trans-bind filter paper (Schleicher and Schuell) were activated by exposure to a freshly prepared solution of NaNO₂ in 12 × HCl for 30 min at 4 °C and washed three times in cold H₂O and also in 0.2 M sodium acetate, pH 4.0, at room temperature. The cDNA insert from clone A1 was denatured, spotted onto the activated paper, and allowed to dry overnight. The filters were then cut into 1-mm squares and washed sequentially with sterile distilled water, 0.4 M NaOH, and then distilled water. Filters were prehybridized for 1 h at 42 °C in a solution containing 60% formamide, 10 mM PIPES, pH 6.4, 0.4 M NaCl, and 20 mg/ml yeast tRNA. The hybridization mixture containing 25 μg of total RNA or poly(A) RNA, 60% formamide, 10 mM PIPES, pH 6.4, and 0.075 M NaCl was added and allowed to hybridize at 42 °C overnight. The filters were extensively washed in 0.5 × SSC, 0.5% SDS at 60 °C and once in 2 mM EDTA, pH 8.0, at room temperature. Elution of hybrid-selected mRNA was achieved by boiling for 90 s in 1 mM EDTA, pH 7.5. The supernatant fraction containing the hybrid-selected mRNA was ethanol-precipitated using calf liver tRNA as carrier. The precipitate was lyophilized and resuspended in 2.2 μl of sterile distilled water for translation in a rabbit reticulocyte lysate system in the presence of [³²P]methionine. Translation was terminated by the addition of a solution of 125 mM Tris/HCl at pH 6.8, containing 5% SDS, 20% glycerol, 0.1 mM β-mercaptoethanol, and 0.02% bromphenol blue; for immunoprecipitation in 0.1 M Tris-HCl, pH 7.2, containing 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 1% Transferr. Polyclonal rabbit antiserum against chick anchorin or normal rabbit serum was used along with protein A coupled to Sepharose beads to bind labeled proteins. These were subsequently released from the beads by boiling in SDS-gel sample buffer and electrophoresed.

RESULTS

Isolation of cDNA Clones for Anchorin CII—Initially, we screened a cDNA library prepared from chick chondrocyte RNA using a polyclonal antibody against chick chondrocyte anchorin (16). Of approximately 10⁷ phages screened, 30 reacted positively with the anti-anchorin antibody and 17 clones remained strongly positive after purification. Six of the clones were related, as demonstrated by Southern analysis using radiolabeled A1 clone as a hybridization probe (data not shown). A restriction map of these clones is shown in Fig. 1. To obtain larger cDNA clones, we screened the library by hybridization with labeled DNA fragments from previously isolated clones and obtained several overlapping clones, two of which (A4 and A6) are shown in Fig. 1. Both extend to the 3′ untranslated regions. The 3′-end of the coding region was confirmed both by nucleotide and by peptide sequencing.

Identification of Anchorin cDNA Clones by Hybrid Selected Translation—We utilized hybrid selection of chondrocyte mRNA with the cloned DNA followed by translation as one method to establish the identity of anchorin. In these studies, the insert from clone A1 was immobilized on activated di-azobenzyloxymethyl paper and allowed to hybridize to chondrocyte poly(A) RNA. Bound mRNA was eluted and translated in a rabbit reticulocyte lysate in the presence of [³²P]methionine. These studies showed that hybrid selection of cartilage RNA by a 780-bp fragment from clone A1 allows the isolation of RNA which is translated predominantly to a single protein (M₀ = 32,000) (Fig. 2A). Furthermore, this protein is immunoprecipitated by anti-anchorin antiserum as shown in Fig. 2B, lanes 3 and 4. The difference in size obtained from the translated protein (M₀ = 32,000), and authentic anchorin (M₀ = 34,000) from chondrocytes on electrophoresis could be due to differences in glycosylation or other post-translational modifications. In spite of the difference in size, however, the

Fig. 1. Restriction map and locations of cDNA clones for chick anchorin. A restriction map of the inserts of anchorin clones was constructed using several endonucleases as described in the text. The number below the bar indicates the distance in base pairs from the 5′-end of the cDNA. The hatched bar from 987 bp to the end represents the 3′-untranslated region.
immunological cross-reactivity of the proteins indicates that they are both forms of anchorin and that clone A1 codes for anchorin.

Northern Analysis—Different portions of the cDNA clones for anchorin were used to analyze the mRNA species in chick cells. The 880-bp EcoRI fragment from the 5' portion of the cDNA hybridized to a single 1.7-kb mRNA species (Fig. 3A). This mRNA is found both in chondrocyte and in normal and Rous sarcoma virus-transformed chick embryo fibroblasts. Interestingly, a 3-fold higher level of anchorin mRNA was found in chondrocyte and in normal and Rous sarcoma virus-transformed fibroblast (lane 3), and crop (lane 4). B, hybridization with 3' probe: sterna (lane 1), fibroblasts (lane 2), Rous sarcoma virus-transformed fibroblast (lane 3), and crop (lane 4). Positions in the gel of 28 S and 18 S ribosomal RNAs are indicated.

Sequence of Anchorin—The nucleotide sequences of the cDNA clones listed in Fig. 1 were determined (Fig. 4). A continuous sequence with a single open reading frame of 987 nucleotides coding for a protein \( M_r = 37,344 \) composed of 329 residues (Fig. 4) was determined. There is no poly(A) tail in the first 250 nucleotides of the 3' untranslated segment. The deduced amino acid sequence does not contain Asn-X-Ser or Asn-Thr-Ser sequences, potential sites for N-linked glycosylation, and Asn-X-Thr sequences, potential sites for N-linked glycosylation. The deduced amino acid sequence does not contain Asn-X-Ser or Asn-Thr-Ser sequences, potential sites for N-linked glycosylation, and Asn-X-Thr sequences, potential sites for N-linked glycosylation. The deduced amino acid sequence does not contain Asn-X-Ser or Asn-Thr-Ser sequences, potential sites for N-linked glycosylation, and Asn-X-Thr sequences, potential sites for N-linked glycosylation.

![Fig. 2. Hybrid-selected translation.](image)

**FIG. 2. Hybrid-selected translation.** A 780-bp EcoRI fragment from clone A1 was immobilized on diazobenzyloxymethylcellulose paper and hybridized to total RNA or to poly(A)+ RNA from chick cartilage. Hybrid-selected RNA was eluted and translated in a rabbit reticulocyte lysate, and the product was electrophoresed in a 10% SDS-polyacrylamide gel. A, translation products: in the absence of RNA (lane 1), from total cartilage RNA (lane 2), from hybrid-selected nitrocellulose paper alone (lane 3), or from mRNA hybrid-selected with clone A1 (lane 4). B, immunoprecipitation with anti-anchorin antiserum of the translation products: in absence of RNA (lane 1), from total cartilage RNA (lane 3), from mRNA hybrid selected with clone A1 (lane 4). Lane 2 represents the control immunoprecipitation with nonimmune rabbit serum of the translation products from total cartilage RNA. Lower arrow points to migration of in vitro-translated anchorin. Upper arrow points the position at which purified anchorin migrated in the same gel.

![Fig. 3. Northern analysis.](image)

**FIG. 3. Northern analysis.** The 5' probe, a 780-bp EcoRI fragment of clone A1, and the 3' probe, a 600-bp EcoRI fragment of clone A4, were labeled by nick translation and hybridized to RNA which had been blotted onto a nitrocellulose membrane after electrophoresis on a denaturing formaldehyde-agarose gel. A, hybridization with 5' probe. Twenty \( \mu \)g of total RNA, from different chick tissues, were blotted on each lane: sterna (lane 1), fibroblasts (lane 2), Rous sarcoma virus-transformed fibroblast (lane 3), and crop (lane 4). B, hybridization with 3' probe: sterna (lane 1), fibroblasts (lane 2), and crop (lane 3). Positions in the gel of 28 S and 18 S ribosomal RNAs are indicated.

![Fig. 4. Nucleotide sequence of the cDNA coding for chick anchorin and the deduced amino acid sequence of the protein.](image)

**FIG. 4. Nucleotide sequence of the cDNA coding for chick anchorin and the deduced amino acid sequence of the protein.** Nucleotides are numbered at the top right and amino acids at the bottom right. The translation into amino acid sequence is shown below as a single open reading frame. Sequences underlined are found to match peptide sequences obtained from anchorin tryptic fragments. The box enclosing residues 244-267 represents the hydrophobic region. The tyrosine residue in parentheses is a possible phosphorylation site. Cysteines in the sequence are circled. The arrowhead at nucleotide 883 points at the EcoRI cleavage site in the anchorin mRNA.
oligosaccharides. However, there are several serine and threonine residues as possible sites for O-linked oligosaccharides. A tyrosine at residue 265 might be a potential phosphorylation site since the sequence around this residue resembles a typical tyrosine phosphorylation signal (31). The COOH-terminal portion of the protein is rich in aspartic acid (9 out of 62 residues), whereas the NH$_2$-terminal portion contains an unusually high amount of glutamic acid (10 out of 68 residues).

Isolation and Sequencing of Peptides from Anchorin CII—Anchorin CII prepared from chicken chondrocyte membranes was subjected to preparative slab gel electrophoresis and a band (Mr = 34,000), identified as anchorin CII by immunoblotting (16), was excised and digested with trypsin. The resulting peptides were eluted from the polyacrylamide gel and separated by reverse-phase chromatography into 13 major fractions (Fig. 5A). Each peak was rechromatographed by reverse-phase chromatography using other conditions (Fig. 5B). Thirteen purified tryptic peptides encompassing 113 residues were sequenced (Table I).

Comparison of Peptide Sequences with the Sequence Predicted from the cDNA—The nucleotide sequence of clone A1 contains an open reading frame of 780 nucleotides which code for 260 residues and include the sequences obtained for the 7 tryptic peptides underlined in Fig. 4. This clone is located 100 bp away from an EcoRI cleavage site within the anchorin CII nucleotide sequence. Additional cDNA clones were obtained from the original λgt11 library by DNA hybridization with nick-translated 3'-end DNA fragments from clone A15 (see Fig. 1). Two of these clones, A4 and A6, were sequenced, and both were identified as anchorin CII, since they contained three other tryptic peptides (Fig. 4). The orientation of these clones as being COOH-terminal was established by the amino acid sequence of an overlapping tetrapeptide (HEFR) (Fig. 4) covering the internal EcoRI site. These clones include the continuation of an open reading frame of 207 base pairs coding for an additional 69 amino acids and a nontranslated region of 248 base pairs. The sequences of 10 out of the 13 peptides are present in the deduced amino acid sequence (underlined in Fig. 4). The sequence of three of the tryptic peptides on Table I is not contained in the sequence depicted in Fig. 4. They could arise from the 5'-end of the sequence or be derived from an alternatively spliced exon which is not present in our cDNA clones.

**DISCUSSION**

We have isolated several overlapping cDNA clones which span 1.5 kb encoding anchorin CII. The identification of the clones was established by hybridization to anchorin CII mRNA. Further, 10 peptides from anchorin have been sequenced and found to occur in the protein predicted by the sequence of our cDNA clones. Anchorin CII thus consists of at least 329 residues (Mr = 37,344), some 10% larger than the size estimated by electrophoresis. Primer extension analysis (data not shown) revealed that the cDNA clones described here contain the 5'-end of anchorin mRNA, suggesting that the methionine at residue 7 could be the initiating residue. Additionally, this methionine residue is implicated as the initiation site since sequences flanking the ATG codon con-

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**TABLE I**

Amino acid sequences derived from trypsin digestion of anchorin CII

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Length</th>
<th>Position in sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Gly-Thr-Val-Thr-Ala-Phe-Ser-Pro-Phe-Asp-Ala-Arg</td>
<td>12</td>
<td>13-24</td>
</tr>
<tr>
<td>2. Ile-Phe-Asp-Ala-His-Ala-Leu-Lys</td>
<td>8</td>
<td>96-103</td>
</tr>
<tr>
<td>3. Gln-Glu-Ile-Ala-Ser-Ala-Phe-Lys</td>
<td>8</td>
<td>57-64</td>
</tr>
<tr>
<td>4. Ser-Val-Ser-His-Leu-Arg</td>
<td>6</td>
<td>210-215</td>
</tr>
<tr>
<td>5. Ser-Leu-Tyr-Gln-Met-Ile-Gln-Lys</td>
<td>8</td>
<td>303-310</td>
</tr>
<tr>
<td>6. Val-Leu-Thr-Glu-Ile-Leu-Ala-Ser-Arg</td>
<td>9</td>
<td>115-123</td>
</tr>
<tr>
<td>7. His-Glu-Phe-Arg</td>
<td>4</td>
<td>294-297</td>
</tr>
<tr>
<td>8. Val-Glu-Ala-Leu-Vai-Glu-Lys</td>
<td>8</td>
<td>175-182</td>
</tr>
<tr>
<td>9. Thr-Pro-Ala-Glu-Val-Gln-Asn-Ile-Lys</td>
<td>9</td>
<td>Not found</td>
</tr>
<tr>
<td>11. Ile-Thr-Gly-Thr-Thr-Ser-Gly-His-Pro-Gln-Arg</td>
<td>11</td>
<td>269-279</td>
</tr>
<tr>
<td>12. Gly-Ala-Gly-Thr-Asp-Asp-Thr-Leu-Ile-Arg</td>
<td>5</td>
<td>65-69</td>
</tr>
<tr>
<td>Totalc</td>
<td>113</td>
<td></td>
</tr>
</tbody>
</table>
tain the critical requirements for initiation of translation, an A in position -3 and a G in position +4 (32). If this is the case, there may not be an amino-terminal signal peptide in anchorin, although there are examples of internal transmembrane segments being utilized as signal peptides (33, 35). The only hydrophobic sequence in anchorin (residues 244–267) contains 3 charged residues, which is unusual for a transmembrane segment. There are several proteins, however, such as glycoporin A (36) and bacteriorhodopsin (37) whose transmembrane segments have charged amino acids. It is possible that anchorin CII may actually be a membrane-associated protein, rather than a transmembrane protein. In the future, antibodies to synthetic peptides corresponding to NH2- and COOH-terminal sequences could be generated and used to establish whether both ends of the protein are outside of the cell.

Northern analysis revealed that the 5’ cDNA probe hybridized to 1.7-kb mRNA species as expected for anchorin, whereas the 3’ cDNA probe hybridized to a 5-kb as well as the 1.7-kb mRNA species. These results suggest that the 1.7-kb and 5-kb mRNAs are transcribed from different genes but contain homologous sequences or that the two transcripts are derived from the same gene via alternative splicing. Many genes have been reported to have undergone alternative splicing to generate a diversity of structures. It is also conceivable that anchorin belongs to a family of proteins and that the 5.5 mRNA species could code for a protein whose sequence is homologous to anchorin. Since some of the amino acid sequences from proteolytic fragments of anchorin are not present in the deduced sequence from cDNA clones, it is possible that they arise from another form of anchorin.

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