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Molecular analysis of chicken embryo SPARC (osteonectin)

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SPARC is a secreted glycoprotein that modulates cell shape and cell-matrix interactions. Levels of SPARC are increased at sites of somitogenesis, osteogenesis, and angiogenesis in the embryo and during wound repair in the adult. We have cloned and characterized SPARC from chicken embryo. A 2.2-kbp cDNA, obtained by a novel use of the polymerase chain reaction, was determined to encode a 298-residue protein that is 85% identical to human SPARC. Antigenic sites in particular appear to be highly conserved, as antibodies against C-terminal sequences of murine and bovine SPARC reacted with a 41–43 kDa protein in chicken embryo extracts. Chicken SPARC can be defined by four sequence signatures: (a) a conserved spacing of 11 cysteine residues in domain II, (b) the pentapeptide KKGHK in domain II, which is contained within a larger region of 31 identical residues, (c) a 100% conserved region of 10 residues in domain III, and (d) a C-terminal, calcium-binding EF-hand motif. SPARC mRNAs in the 10-day-old chicken embryo are represented by three sizes of 1.8, 2.2 and 3.0 kb. The relative steady-state levels for the 2.2-kb mRNA were determined as aorta ≥ skeletal muscle > calvarium > vertebra > anterior limb > kidney > heart > brain > skin and lung ≫ liver. The relative abundance of the 1.8-kb and 2.2-kb mRNAs varied among tissues and indicated that differential processing of SPARC mRNAs might occur. All three RNA species were detected by a cDNA probe for the N-terminal part of the coding region. Thus, the three mRNA species appear to arise from differential 3′ splicing and/or polyadenylation. Collective evidence demonstrates that SPARC has been well-conserved during vertebrate evolution, a finding that indicates a fundamental role for this protein in development.

SPARC (secreted protein acidic and rich in cysteine) is a highly-conserved protein among tissues and vertebrate species. The protein is a product of many cultured cells but is characteristically produced *in vivo* by tissues undergoing morphogenesis and remodeling (Sage et al., 1986, 1989a, b, 1992; Holland et al., 1987). SPARC was originally described as osteonectin, an abundant component of the extracellular matrix in bone (Termine et al., 1981). The identity of these two proteins became apparent with the molecular cloning of the mouse (Mason et al., 1986), bovine (Bolander et al., 1988) and human cDNAs (Villarreal et al., 1988). SPARC is also known as the basement membrane protein BM-40 (Dziadek et al., 1986; Lankat-Buttgereit, 1988), which binds to collagen types I–V in a calcium-dependent manner (Sage et al., 1989a; Lane and Sage, 1990; Mayer et al., 1991). Many cell types (normal and transformed) and tissues of diverse embryonic origin express and secrete the product of this single-copy gene.

The active sites and known activities of SPARC are best described by an examination of the domain structure of the protein. There is general agreement among investigators using either purified vertebrate SPARC (Sage and Bornstein, 1991), purified vertebrate BM-40 (Timpl, 1989), synthetic SPARC peptides (Lane and Sage, 1990; Funk and Sage, 1991, 1993; Hasselaar and Sage, 1992), purified BM-40 fragments (Mayer et al., 1991), or recombinant BM-40 (Nischt et al., 1991). The collective data indicate a four-domain structure for this extracellular protein (Lankat-Buttgereit et al., 1988; Bolander et al., 1988; Engel et al., 1987). The N-terminal domain I contains elements which modulate cell shape and prevent the spreading of cultured endothelial cells (Lane and Sage, 1990). Domain II contains sequences that affect cell proliferation (Funk and Sage, 1991, 1993). Domain III contains a major cleavage site for serine proteases (Mann et al., 1987) and the carboxy-terminal fourth domain contains both a Ca^{2+}-binding EF-hand (Engel et al., 1987) that is involved in the binding of SPARC to collagen types I–V and sequences which modulate cell shape (Lane and Sage, 1990).

A broad tissue distribution indicates diverse functions for the protein. It is likely that SPARC accommodates its many functions through activities localized to specific domains and regions. Synthetic peptides have proven useful to define these activities (Lane and Sage, 1990). A 10-residue synthetic peptide, termed peptide 2.1a and derived from sequences within the second domain of SPARC, retarded cell-cycle progression of bovine endothelial cells (Funk and Sage, 1993). In contrast, significantly lower concentrations of an 18-amino-acid synthetic peptide, termed peptide 2.3, stimulated the incorporation of [^{3}H]thymidine by endothelial cells in a dose-dependent manner (Funk and Sage, 1993). Since

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Abbreviations. PCR, polymerase chain reaction; EGF, epidermal growth factor; SPARC, secreted protein acidic and rich in cysteine.

Note. The novel amino acid sequence data published here have been submitted to the Genbank sequence data bank and are available under accession number L24906.

native SPARC displays only the inhibitory activity of peptide 2.1a, and not the growth-promoting properties observed with peptide 2.3, we have proposed that specific proteolysis of the protein is necessary, e.g., to unmask the mitotic effect of SPARC on endothelial cells (Lane, T., Iruela-Arispe, M. L., Johnson, R. and Sage, E. H. unpublished results). Inherent in this prediction is a conservation of such proteolytic sites in SPARC proteins from different species. Since SPARC has been well-conserved among vertebrates, many investigators have used cDNAs from one species as probes to analyze the RNA from different species; this strategy has generally proved reliable in Northern analyses. However, two recent studies (Johnston et al., 1990; Guermah et al., 1991) have indicated that SPARC-like proteins displaying a remarkably high level of amino acid sequence identity with SPARC are present in rodent and avian species. We were therefore interested in whether the mRNA size and amino acid sequence of chicken SPARC were conserved with respect to their human, bovine and rodent counterparts.

In this study, we used a novel polymerase-chain-reaction (PCR) procedure to assemble the cDNA and deduced amino acid sequence for chicken embryo SPARC. Multiple sequence alignments of mammalian, amphibian and avian SPARC demonstrate that the protein has been highly conserved throughout vertebrate evolution. The results from database comparisons indicate that the C-terminal region of SPARC (two-thirds of the intact protein) is significantly similar to the two SPARC-like proteins from quail retina and rat brain. Northern-blot analysis of RNA from 14 chicken embryo tissues with cDNA revealed three discrete mRNAs which appear to undergo differential processing within tissues.

MATERIALS AND METHODS

Isolation of SPARC cDNA fragments by the polymerase chain reaction

The first oligonucleotide primer was derived from domain 2.3, a conserved region of SPARC. This oligonucleotide primer [1F: 5′d(CACAAGCTCACCTGGACTACATC)3′; T_m = 64°C] was paired with an oligonucleotide primer specific for the sequence of the left arm of λgt11 [1R: 5′d(GACACCAGACCAACTGGTAAT)3′, T_m = 56°C]. Total DNA was isolated from a 15-day-old chicken embryo λgt11 cDNA library and was used as a template in PCR. The oligonucleotide primers 1F and 1R were annealed (step 1) to 0.1 μg total library DNA and 30 cycles of PCR were performed (94°C, 1 min; 58°C, 1 min; 72°C, 1 min). Dideoxynucleotide chain-termination sequencing revealed that this PCR DNA product was derived from chicken embryo SPARC mRNA. A second oligonucleotide primer was designed from the 5′-end of the above PCR DNA product [2R: 5′d(GTGTCCAGGCAGGCAGGGATGAATITG)3′; T_m = 74°C]. This oligonucleotide primer was paired to an oligonucleotide primer specific for the sequence of the right arm of λgt11 [2F: 5′d(GGTGGCGACGACTCCTGGAGC)3′, T_m = 71°C], annealed (step 2) to 0.1 μg total library DNA, and amplified by *Taq* polymerase as described above.

A single PCR DNA product encoding the secreted form of chicken embryo SPARC was obtained through the use of two additional oligonucleotide primers [3F: 5′d(GATATACATATGGCTCCGCAAGAGGCTCTGGCGC)3′, T_m= 81°C; 3R: 5′d(ACCACCTCGAGTTAGATCACCAGATCCTTGTCTATG)3′, T_m = 74°C]. These oligonucleotide primers were annealed to 0.1 μg total library DNA and a 842-bp DNA product was obtained from 28 cycles of PCR (94°C, 1 min; 62°C, 1 min; 72°C, 1 min). This DNA was ligated to the plasmid vector PCRII (Invitrogen) through A-T ends. This recombinant plasmid was termed rSPARC-pCRII. The primers 3F and 3R contain the recognition sequences for the restriction endonucleases *Nde*I and *Xho*I, respectively.

Sequence determination by oligonucleotide primer walking

The PCR DNA products were cloned into plasmid vectors and sequenced by dideoxynucleotide chain-termination methods with [^{35}S]dATP[S] and a modified form of T7 DNA polymerase (Sequenase, US Biochemicals). Both single-stranded and double-stranded rSPARC-pCRII DNA were used as templates for the complete sequence determination of both strands. The sequence-specific oligonucleotide primers were synthesized as needed to extend the sequence.

Northern blotting

Fertilized white leghorn chicken eggs were incubated at 37°C and 60% relative humidity for ten days. The eggs were opened and the embryos were placed in sterile Dulbecco's modified Eagle's medium. The following organs and tissues were removed with the aid of a dissecting microscope: calvarium, eye, intestine, skin, lung, vertebra, liver, bone, kidney, anterior limb, brain, skeletal muscle and aorta. Total RNA was isolated by the procedure described by Chomczynski and Sacchi (1987) as modified by Puissant and Houdebine (1990). 10 μg total RNA was fractionated on a 1% agarose gel that contained 2.2 M formaldehyde, transferred to nylon membranes (Nytran) by vacuum blotting and pre-hybridized as described (Iruela-Arispe et al., 1991). A *Nde*I–*Xho*I fragment of rSPARC-pCRII was gel-purified, radiolabeled with [^{32}P]dCTP ($0.3\times10^9-1\times10^9$ cpm/μg DNA) and denatured. The probe was added to fresh hybridization buffer and the mixture was incubated with the membrane at 42°C for 16 h. As a control for loading and transfer efficiency, all membranes were hybridized with a 28S rRNA fragment (Iruela-Arispe et al., 1991). For quantification, several exposures of Northern blots were processed by scanning densitometry. The values were normalized to the 28S rRNA signal.

Immunoblotting

Tissue and cell culture media were assayed for SPARC by SDS/PAGE followed by immunoblotting. Specific, polyclonal antibodies produced against mouse SPARC and synthetic peptide 4.2 were used (Lane and Sage, 1990). Antibodies were used directly or diluted to 2 μg/ml and incubated with either 50 μM peptide 4.2 or 10–20 μg/ml brain extract for 2–16 h at 4°C. The absorbed antibodies were further diluted to 1:100 prior to use. Protein samples were collected from the media of cultured chicken smooth muscle cells or by homogenization of dissected tissue from 10-day white leghorn chicken embryos with 0.1% Nonidet P-40, 50 mM Tris/HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 100 mM *N*-ethylmaleimide, 10 mM pepstatin A and 5 mM 1,10-phenanthroline. The samples were dissolved in SDS/PAGE sample buffer (0.125 M Tris/HCl, pH 6.8, 2–5% SDS, 0.1 M dithiothreitol, 5% glycerol, 0.01% bromophenol blue) and were heated to 95°C for 5–10 min. Alternatively,

tissue extracts were incubated with 50 mM dithiothreitol for 60 min at 37°C, and subsequently incubated with a fourfold molar excess of iodoacetic acid for 60 min at 25°C. Proteins were fractionated by 10–20% gradient polyacrylamide electrophoretic gels that contained 0.1% SDS and were electrotransferred to nitrocellulose membranes (0.45-μm pore size). The bound protein-immunoglobulin complexes were detected with ^{125}I-labeled protein A and were visualized by autoradiography.

Chicken embryo smooth muscle cell cultures

The conditioned medium was harvested from cells of the aortic wall of a 15-day-old chicken embryo. Cells were seeded at 5×10^5 cells/35-mm dish. 24 h after initial culture, the cells were washed and incubated overnight in minimal essential medium. The medium was subsequently collected and centrifuged at $750\times g$ for 10 min to remove debris. After the addition of phenylmethylsulfonyl fluoride to a final concentration of 0.2 mM, the medium was stored at −70°C prior to use.

Sequence and secondary-structure analyses. Database comparisons and pattern recognition

The primary structures of SPARC proteins were analyzed with a collection of programs written by the Genetics Computer Group (Madison, Wisconsin; Devereux et al., 1984). A profile was constructed from aligned protein sequences and used to search through the Swiss Prot (Release 23.0, August 1992) and GenPept databases (Release 72.0, June 1992) for sequences related to SPARC. Selected results from PROFILESEARCH, length of polypeptide and Z score, were determined and plotted separately to create a graphic representation of the SPARC profile of sequences. The sequence comparisons and similarity were calculated using FASTA. The functional motifs were identified from a search of the ProSite database.

Predictions of the hydrophilicity of the complete, derived amino acid sequence for chicken embryo SPARC were calculated according to the algorithm of Hopp and Woods (1981) through the computer program PEPTIDESTRUCTURE (Jameson and Wolf, 1988). The results of this analysis were compared to those obtained by the method of Kyte and Doolittle (1982). No major differences were observed between the two algorithms. The predictions of α-helicity used the algorithms of Chou and Fasman (1978) and Garnier et al. (1978).

RESULTS

Isolation of SPARC cDNA fragments by the polymerase chain reaction

We used a novel PCR procedure to isolate cDNA fragments that encoded chicken embryo SPARC. An analysis of a multiple sequence alignment of cDNA sequences from human, mouse and bovine SPARC revealed that a central region of the molecule, domain 2.3, had been completely conserved at the DNA level. We subsequently designed an oligonucleotide primer derived from this conserved region to pair with an oligonucleotide primer specific for the sequence of the left arm of λgt11. Total DNA was isolated from a chicken embryo λgt11 cDNA library and was subsequently used as a template in PCR. The oligonucleotide primers 1F and 1R were annealed to 0.1 μg total library DNA and PCR was performed (step 1). Agarose gel electrophoresis identified a band of 1600 bp (data not shown), a value in excellent agreement with the size predicted from the human, mouse and bovine SPARC cDNAs. Subsequent dideoxynucleotide chain-termination sequencing indicated that this cDNA fragment was derived from chicken embryo SPARC mRNA.

A second oligonucleotide primer was designed from the 5′-end of the above PCR DNA product. This oligonucleotide primer was paired with an oligonucleotide primer specific for the sequence of the right arm of λgt11, annealed (step 2) to 0.1 μg total library DNA and processed by PCR as described above. Subsequent dideoxynucleotide chain-termination sequencing of the resultant 540-bp PCR DNA product confirmed that this DNA contained 3′ sequences that overlapped 5′ sequences of the first PCR DNA product. The compilation of these two sequences completed the cDNA sequence determination for chicken embryo SPARC. To verify the fidelity of *Taq* polymerase in the generation of a sequence template for chicken SPARC, a single PCR DNA product that encoded the mature, secreted form of chicken embryo SPARC was obtained through the use of two additional oligonucleotide primers. These oligonucleotide primers were annealed to 0.1 μg total library DNA and a 958-bp DNA product was obtained from 28 cycles of PCR. The subsequent cloning and dideoxynucleotide chain-termination sequencing of both strands of the resultant recombinant plasmid, termed rSPARC-pCRII, confirmed our first DNA sequence for the mature, secreted form of chicken embryo SPARC.

Primary structure of chicken embryo SPARC

The predicted amino acid sequence of the avian protein is shown in Fig. 1. A 17-residue, N-terminal signal peptide was identified based on its characteristic hydrophobic amino acid content and by comparison with the signal peptides and primary structures of human, mouse, bovine and frog SPARC. The remaining 281 amino acid residues comprise the secreted form of the protein, which has a calculated molecular mass of 32.09 kDa. Chicken SPARC is an acidic molecule with a predicted isoelectric point of 4.50.

Profile analysis and amino acid sequence comparisons

The deduced N-terminal amino acid sequence of chicken embryo SPARC was compared with the Edman-degradation sequence of SPARC isolated from adult chicken bone (data not shown; Findlay et al., 1988). A match of 30 out of 32 residues was observed. Although the other SPARC proteins begin with APQQ at their N-termini, chicken embryo SPARC begins with APQE. The lack of a glutamine residue at position 3 is inconsistent with the sequence derived from the Edman degradation of SPARC from chicken bone. We note that our sequence was derived from three independent PCR reactions that yielded identical results. The discrepancies between chicken embryo SPARC, derived from PCR analysis in the present study, and of adult chicken SPARC, derived from the Edman degradation of bone SPARC, might have arisen from the use of different strains of *Gallus gallus*, or from genetic polymorphism. The majority of sequence divergence is seen at the N-terminus (Fig. 1), which contains sequences within exon 3 of the SPARC gene (Findlay et al., 1988).

We used profile searching to characterize a multiple alignment of the five known SPARC amino acid sequences,

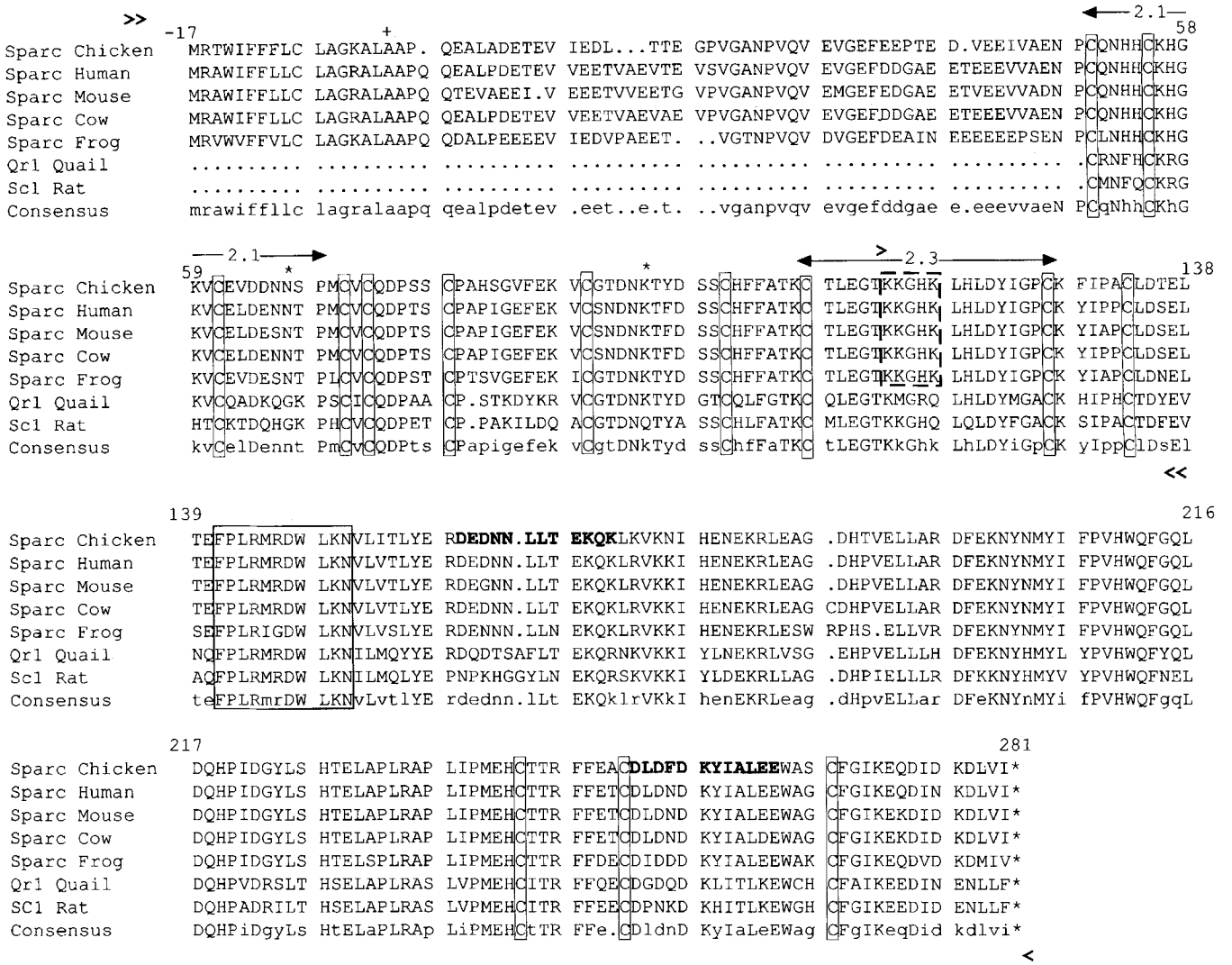

Fig. 1. Amino acid sequence alignment of chicken SPARC, vertebrate SPARC proteins and SPARC-like proteins. The amino acid sequence was deduced from the cDNA sequence. The mature form of the sequence is Ala1−Ile281. The N-terminal signal peptide is from −17 to −1. Regions that correspond to synthetic peptides 2.1 and 2.3 are indicated (↔). KHG and KKGHK are in boldface type. The oligonuocleotide primers (> < and >> <<) used to assemble the cDNA sequence by PCR are indicated. Putative EF-hand signatures (boldface) are located at residues 160−171 and at 252−263. Potential N-linked glycosylation sites are at Asn67 and Lys95 (*). The sequences were obtained from either the Swiss-Prot or Protein Identification Resource database and aligned with PILEUP. The bottom strand is a consensus sequence in which capital letters indicate a match in all seven proteins. A lower-case letter is the predominant amino acid at that position. The lack of a consensus residue in the consensus strand is indicated (•). Conserved cysteine residues and an 11-residue signature region are boxed. The putative Cu^{2+}-binding motif, KKGHK, is boxed with a dotted line.

because the alignment of a sequence to a profile is inherently more sensitive than pairwise alignment algorithms. The complete, primary structure of chicken embryo SPARC was aligned with sequences that corresponded to human, bovine, mouse and frog SPARC with PILEUP, a multiple sequence alignment computer program (Fig. 1). The sequence similarities based on comparisons of the mature, secreted protein are as follows: chicken versus (human, mouse, bovine) = 85% and chicken versus frog = 78%.

A profile analysis of all the aligned sequences is shown in Fig. 2. The five SPARC sequences (chicken, human, mouse, bovine and frog) are located at $x = 302-304$, $y = 67-69$, with three coordinates superimposed upon each other. The analysis also identified two related polypeptide sequence entries: (a) embryonic quail retinal cells express an mRNA encoding a 676-residue protein termed QR1 ($x = 676$, $y = 29$) that is 58% identical to chicken SPARC (Guermah et al., 1991); (b) the C-terminal 270 amino acids of a 634-residue protein termed SC1 ($x = 634$, $y = 28$) show 55% identity with chicken SPARC. The primary structure of rat brain SC1 was determined from its mRNA sequence (Johnston et al., 1990).

Pattern recognition of sequence motifs

A closer inspection of the data in Fig. 2 indicates several sequence entries which display statistically significant Z scores of 6−8. When these entries are aligned against chicken embryo SPARC, several interesting features of the chicken sequence become apparent.

Post-translational modifications

Chicken embryo SPARC contains two consensus sites for the attachment of N-linked carbohydrate chains (Fig. 1,

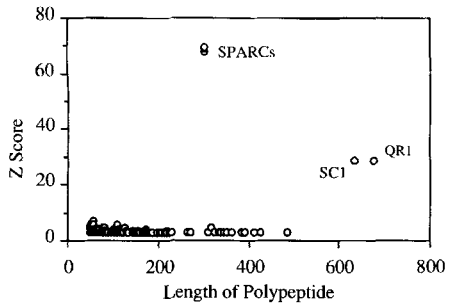

Fig. 2. Profile analysis of SPARC in Swiss Prot database. Statistically significant sequence similarities exceed a Z score of six (Gribskov et al., 1990). Data points representing SPARC from five species, SC1 and QR1 are identified. The background of the databases is observed along the *x* axis.

Asn66–Ser68 and Asn94–Thr96). Human and bovine SPARC each have two glycosylation sites, whereas the mouse and frog proteins have only one site. Potential sites for phosphorylation by protein kinase C (Thr113–Lys115, Thr167–Lys169 and Thr244–Arg246), by casein kinase II (Thr155–Glu158 and Ser 226–Glu229), or by tyrosine kinase (Arg196–Tyr202) are also present (data not shown). SPARC was reported to be phosphorylated (Uchiyama et al., 1986), and Engel et al. (1987) subsequently demonstrated the presence of phosphoserine and phosphotyrosine.

Serine-proteinase-inhibitor region

The data points of $x = 6.2–6.7$, $y = 54$ (Fig. 2) are identified as pancreatic secretory trypsin type I inhibitors. Residues Val89–Cys127 are most similar (38% for 48 amino acids) to avian ovomucoids (Kato et al., 1987; Laskowski et al., 1987). Proteinase-inhibitory activity has yet to be attributed to SPARC (Sage et al., 1984; Hasselaar and Sage, 1991).

Epidermal-growth-factor-like domain

The epidermal-growth-factor (EGF)-like pattern of amino acid sequence is present within chicken embryo SPARC (Cys50–Cys79, Fig. 1). The functional significance of the EGF domains in what appear to be unrelated proteins is not yet clear. However, a common feature is that these repeats are found in the extracellular domain of membrane-bound proteins or in proteins known to be secreted. This domain is characterized by a conserved spacing of five cysteine residues.

Ca^{2+}-binding motifs

A search of the ProSite database revealed that two regions of chicken SPARC, Asp160–Lys171, present within domain III and Asp252–Glu263, present within domain IV (Fig. 1), are potential Ca^{2+}-binding loops of EF-hand structures found in calmodulin, troponin C, parvalbumin and related Ca^{2+}-binding proteins of the same evolutionary family (Kretsinger, 1987). Such Ca^{2+}-binding regions consist of a 12-residue loop flanked on both sides by a 12-residue α-helical domain. Both loops of chicken embryo SPARC are flanked by stretches of at least 12 residues capable of forming an α helix. However, we observed that the confidence of α-helical prediction for the residues N-terminal to the first loop were algorithm dependent. Only the second chicken SPARC EF-hand (Asp252–Glu263) was verified to contain adjacent 12-residue α-helices on either side by a variety of algorithms that predict secondary structure (Chou and Fasman, 1978; Garnier et al., 1978). Ca^{2+}-binding sites are also located in domain I: two segments of 14–16 residues, each of which contains 5–7 glutamic acid residues in short clusters, impart a negative charge at physiological pH. These N-terminal clusters of glutamic acids have been implicated in the low-affinity binding of Ca^{2+} (Engel et al., 1987; Maurer et al., 1992).

The Cu^{2+}-binding region is conserved

Cupric ion is known to bind to SPARC (Vernon and Sage, 1989). We noted that the Cu^{2+}-binding tripeptide, GHK (Pickart and Lovejoy, 1987), was present in chicken SPARC (residues Gly116–Lys118). A search of the Swiss-Prot protein sequence database indicates that 563 proteins contain GHK, although in most cases it is unknown whether these proteins bind Cu^{2+}. Chicken SPARC, however, contains the sequence KKGHK (Funk and Sage, 1993; Fig. 1, residues 114–118), a pentapeptide found in only three other protein sequence entries in the Swiss-Prot database. Human, mouse, cow, chicken and *Xenopus* SPARC all contain KKGHK. This remarkably high degree of conservation indicates that the Cu^{2+}-binding region is most likely critical to the function of SPARC.

SPARC domain signatures

Our analysis of seven sequences presented in Fig. 1 resulted in the assignment of three unique domain signatures that characterize this gene product. The first signature consists of approximately 90 residues that contained a perfectly conserved spacing of 11 cysteine residues (Fig. 1). This cysteine-rich sequence, a hallmark of SPARC, also extends to other gene products, namely SC1 and QR1. The second signature is the pentapeptide, KKGHK (Lys114–Lys118, Fig. 1). This Cu^{2+}-binding pentapeptide is found in all five SPARC sequences. KKGHK is contained within a larger signature sequence of 31 identical SPARC amino acids. In contrast, the rat SC1 protein has KKGHQ, with a positively charged amino acid conserved in the fifth position. The third signature is located at (Phe141–Asn151, Fig. 1). This 10-amino-acid region is conserved among SPARC-related glycoproteins, QR1 and SC1 (Fig. 1). Although the frog sequence contains two discrepancies, the avian and mammalian sequences are completely conserved. The fourth signature, which is not unique to SPARC, is a Ca^{2+}-binding EF-hand found in all SPARC proteins. The evolutionary basis of this Ca^{2+}-binding region appears to have arisen from a mechanism other than exon shuffling from the traditional calmodulins or troponin C proteins (McVey et al., 1988).

Secondary structure predictions

Hydrophilicity

The deduced amino acid sequence of chicken SPARC was analyzed with computer algorithms of hydrophilicity and secondary structure. Fig. 3 shows the distribution of hydrophilic amino acids within chicken SPARC. The N-terminus

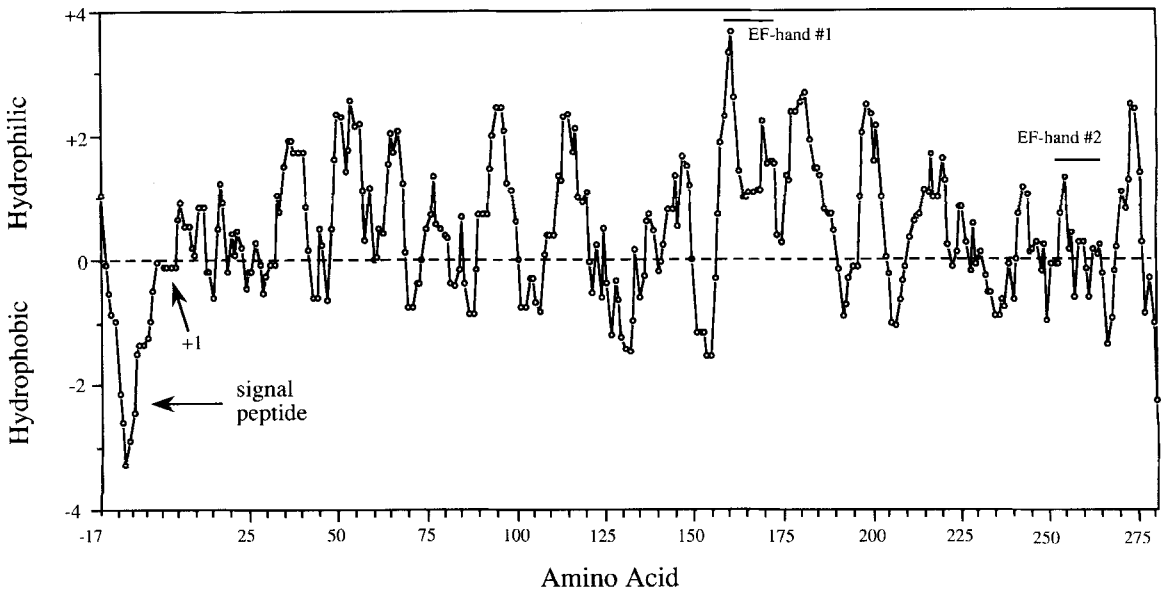

Fig. 3. Distribution of hydrophilic and hydrophobic residues in chicken SPARC. Positive and negative integers along the ordinate represent relative hydrophilicity and hydrophobicity, respectively. The location of the putative signal peptide, signal-peptide-cleavage site and EF-hand regions are indicated.

was shown to contain an extremely hydrophobic sequence of 17 amino acids (Fig. 3), consistent with the presence of a secretory signal.

Domain structure

The mature protein can be divided into four domains as previously described (Engel et al., 1987). An N-terminal domain I (Ala1−Pro49) is rich in glutamic acid and aspartic acid residues (16 out of 49 residues) and in hydrophobic residues (valine, leucine and phenylalanine, 10 out of 49 residues). There are no positively charged residues throughout this domain. A positively charged domain II (Cys50−Cys133) contains 11 out of 15 cysteine residues and 6 of the 7 serine residues of the protein. Domain III (Lys134−Gly187) contains many hydrophilic amino acids and two regions of α-helical structure which are devoid of proline and cysteine (Lys143−Arg158 and Lys172−Gly187). There is an almost equal distribution of negatively and positively charged residues. Domain IV is connected to domain III through a non-α-helical sequence of 31 residues that contains five prolines. The fourth domain also contains the remaining three cysteine residues of the secreted protein, half of the total hydrophobic residues of the avian polypeptide, and several sequences predicted to be α-helical.

Distribution of SPARC protein in embryonic tissues

SPARC is known to be a secreted product of many cultured cells. We detected SPARC in cultured cells derived from chicken embryo smooth muscle. Antibodies against synthetic SPARC peptide 4.2 reacted with a 43-kDa species in Western immunoblots (Fig. 4B); no other immunoreactive proteins were detected. This single band thus served as a positive control when we next examined the immunoreactive proteins from chicken tissues and organs.

Based on studies of mammalian embryos (Holland et al., 1987; Sage et al., 1989b), SPARC was expected to be present in a variety of chicken embryo tissues and organs. We did not know, however, the size of the protein or its potential proteolytic fragments that might arise during development. Tissue extracts were fractionated by SDS/PAGE, transferred to nitrocelluose membranes and probed with different antibodies against SPARC. Fig. 4B shows the result of a representative immunoblot. Antibodies against synthetic SPARC peptide 4.2, a C-terminal region that includes the EF hand (Lane and Sage, 1990), identified a 41−43-kDa immunoreactive protein by comparison with globular protein standards. The chicken protein migrated slightly faster than a purified murine SPARC standard (Fig. 4B, lane 8). The apparent molecular mass of SPARC on SDS/PAGE was 9−11 kDa higher than the predicted size of 32.09 kDa. This retarded electrophoretic mobility is attributed to the covalent attachment of complex-type carbohydrate chains rich in sialic acid and fucose (Hughes et al., 1987). The highest levels of SPARC protein were detected in heart/aorta, lung, limb and calvarium (Fig. 4B, lanes 1, 2, 5 and 6, respectively). No 41−43-kDa immunoreactive bands were detected in brain or liver.

All tissue extracts exhibited immunoreactive bands at 80−90 kDa and at 150−170 kDa. Brain extracts contained an additional, unique polypeptide of 105−110 kDa (Fig. 4B, lane 4). Initial incubation of the antibodies with excess peptide 4.2 resulted in the elimination of the 41−43-kDa immunoreactive species from all samples (data not shown), and of the 110-kDa species from brain. Incubation of anti-(peptide 4.2) immunoglobulins with brain extract followed by subsequent immunoblot analysis eliminated the 25-kDa and 110-kDa bands observed in brain but failed to reduce the intensity of the 80−90-kDa or 150−170-kDa bands (data not shown); a specific reaction between epitopes of the 25-kDa and 110-kDa bands and anti-(peptide 4.2) immunoglobulins was therefore indicated. We also considered whether these higher-molecular-mass bands resulted from intermolecular cystine formation. The sequential reduction and alkylation of extracts with dithiothreitol and iodoacetic acid did not affect

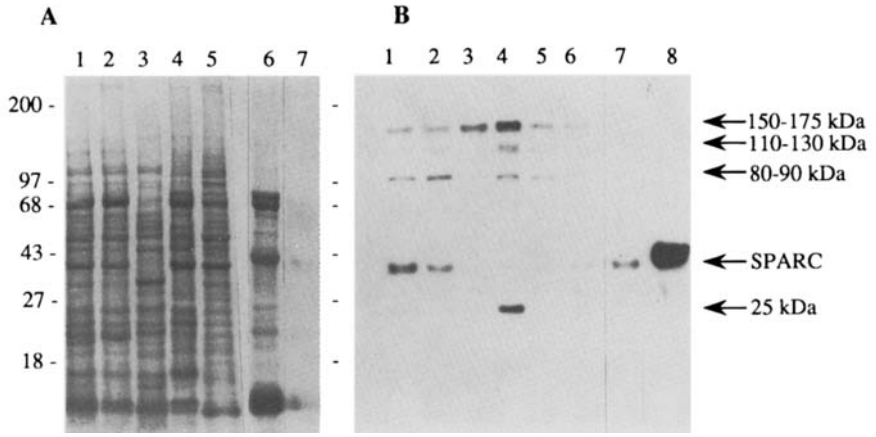

Fig. 4. Immunoreactive SPARC protein in chicken tissues. Tissue or cell culture proteins were fractionated by electrophoresis using 4–15% polyacrylamide gels that contained 0.1% SDS. (A) Coomassie-brilliant-blue-R staining pattern. The apparent molecular masses of marker proteins are indicated (kDa). (B) Autoradiograph of Western immunoblot with anti-(peptide 4.2) immunoglobulins. Bound antigen-antibody complexes were detected with ^{125}I-labeled protein A. Presented is an autoradiograph from a 48-h exposure. Lane 1, heart/aorta; lane 2, lung; lane 3, liver; lane 4, brain; lane 5, limb; lane 6, calvaria; lane 7, smooth muscle cell culture media; lane 8, mouse SPARC (0.5 μg). The immunoreactive components of 80–90 kDa and 150–170 kDa were not diminished by an initial incubation of anti-(peptide 4.2) immunoglobulins with SPARC peptide 4.2 and thus are likely to represent non-specific binding of unidentified chicken proteins.

the migration of the high-molecular-mass components (data not shown).

Structure and expression of SPARC mRNA in the chicken embryo

We observed that the cDNA encoding chicken embryo SPARC is a 2.2-kb species, a value in agreement with the molecular size of SPARC mRNAs from mouse (Mason et al., 1986), human (Swaroop et al., 1988; Villarreal et al., 1989) and cow (Bolander et al., 1988). This molecular size was also reflected in the Northern-blot analysis of RNA extracted from a variety of 10-day-old chicken embryonic tissue (Fig. 5). A methionine codon at base 42, indicative of the initiation signal for protein translation, is followed by an open reading frame of 298 amino acids that extends to a TAA termination codon; the minimum RNA length required to code for SPARC is 894 bp.

The 5′ untranslated region of the cDNA coding strand was approximately 50 bp in length. The length of this region is consistent with the structure of SPARC cDNAs from human, mouse and cow. We examined this portion for CCTG repeats identified by Swaroop et al. (1988) within the 5′ untranslated region of human (seven repeats) and mouse (four repeats) SPARC mRNA. We found that the bovine, chicken, and frog 5′ regions contained four, one and one CCTG repeats, respectively. CCTG repeats are more prevalent in mammalian species and might play a role in translation through the provision of a binding site for regulatory proteins (Swaroop et al., 1988; Findlay et al., 1988). The 3′ untranslated region of 2.2-kb chicken embryo SPARC mRNA is over 1 kb in length. We have identified an AUAAA sequence 28 bases 5′ to the poly(A) tail, which is likely to provide a recognition site for an RNA endonuclease in the polyadenlyation of the SPARC mRNA.

Northern-blot analysis (Fig. 5) also revealed two additional RNA species (3.0 kb and 1.8 kb) which hybridized to a *Nde*I–*Xho*I DNA fragment encoding the secreted form of chicken SPARC. A 2.2-kb and 3.0-kb RNA have also been observed in human tissues (Swaroop et al., 1988). The 1.8-kb RNA appears to be a related nucleic acid because detection persists after stringent hybridization and washing conditions. All three RNA species were detected by a *Nde*I–*Avi*II DNA fragment encoding domains I and II of SPARC. This probe could theoretically hybridize with RNAs encoding the chicken analogs for brain SC1 and retinal QR1. We consider it unlikely that the 3.0-kb RNA detected in chicken tissues represents a QR1 analog, since the distribution of 3.0-kb QR1 RNAs was restricted to embryonic quail retina (Guermah et al., 1991). However, the chicken 3.0-kb RNA species is a candidate for the 3.2-kb SC1 RNA in rat brain and heart (Johnston et al., 1990), as the relative steady-state levels of SC1 RNA are elevated in rat brain (Johnston et al., 1990) and SC1 cDNA is 67% identical to chicken SPARC cDNA. The weak hybridization signal for the chicken SPARC 3.0-kb RNA could be due to the presence of rare copies of this RNA or to the lack of extended regions of complimentary sequences between this RNA and our cDNA probe.

The relative abundance of all three RNA species among 14 tissues and organs was determined by densitometric scanning of autoradiographs from Northern blots (Fig. 5B). Quantification was achieved by normalization to the 28S ribosomal RNA signal. The 3.0-kb RNA transcript was the lowest signal observed. The relative steady-state levels for the 2.2-kb RNA were determined as aorta ≥ skeletal muscle > calvarium > bone > vertebra ≥ anterior limb ≥ eye > heart > kidney > brain and intestine > lung ≫ liver. The relative levels for the 1.8-kb RNA were determined as aorta ≥ skeletal muscle > calvarium > bone > vertebra ≥ eye > anterior limb ≥ heart > lung > skin > intestine > brain > kidney ≫ liver. The levels of the 3.0-, 2.2- and 1.8-kb RNAs varied in relative abundance within each tissue or organ that we examined. The RNAs might therefore have arisen by differential processing by the various tissues.

DISCUSSION

In this study we have isolated a complete cDNA encoding chicken embryo SPARC and have predicted the primary structure of this 298-residue protein. Comparative sequence

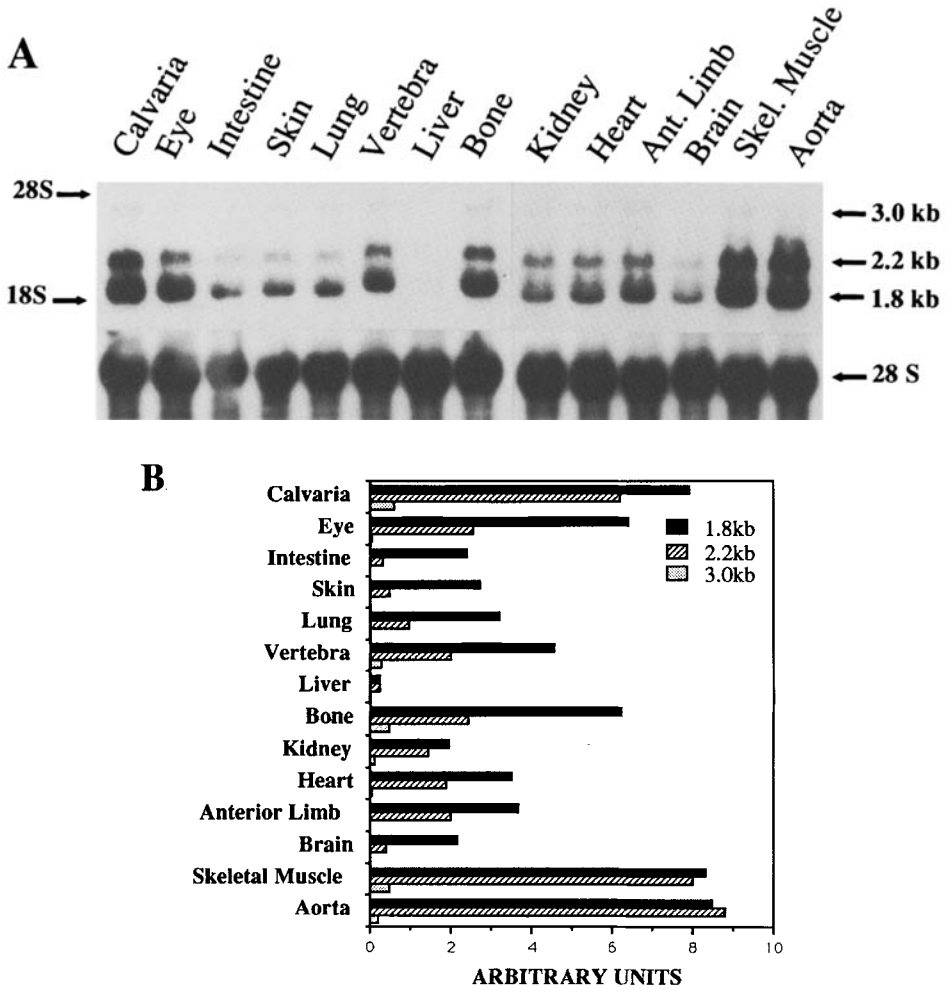

Fig. 5. Relative steady-state levels and tissue distribution of chicken SPARC RNA. (A) 10 μg total RNA from a variety of chicken embryonic tissues was resolved on an agarose gel that contained 2.2 M formaldehyde, transferred to a nylon membrane and hybridized to a cDNA fragment encoding chicken SPARC. Three transcripts (indicated by arrows) were observed of 3.0, 2.2 and 1.8 kb. The migration of the 28S and 18S subunits is indicated (left side). After the removal of bound probe, the membranes were reprobed with a cDNA fragment encoding the bovine 28S ribosomal subunit. Ant., anterior; Skel., skeletal. (B) Relative abundance of the 3.0-, 2.2- and 1.8-kb transcripts was determined by scanning densitometry. Values were corrected for loading by normalization of the absorbance values of the 28S ribosomal subunit and were subsequently plotted as a histogram.

analyses indicate that SPARC has been highly conserved among human, bovine, murine, avian and amphibian species. In addition, regions of two larger proteins, QR1 from quail neuroretina and SC1 from rat brain, display significant sequence similarity to SPARC. Multiple sequence alignments of the five SPARC proteins with QR1 and SC1 demonstrate that these extracellular proteins contain a conserved region of about 240 amino acids that is comprised of three sections with the following features: (a) an N-terminal section of approximately 90 residues that contains 11 conserved cysteines, (b) a central α-helical section and (c) a C-terminal region that contains a Ca^{2+}-binding EF-hand motif and three conserved cysteines. The spacing of the 11 cysteines is a key element in the identification of SPARC and SPARC-like proteins. These sequence patterns also serve to reinforce the traditional four-domain structure of SPARC (Engel et al., 1987) and provide useful insights into the secondary structure of this extracellular protein.

Chicken SPARC contains an unambiguous Ca^{2+}-binding motif of the EF-hand type in the C-terminal domain IV. Others have shown that this motif represents a reversible, high-affinity binding site for Ca^{2+} (K_d = 0.6 μM; Maurer et al., 1992), that this region confers Ca^{2+}-dependent binding of SPARC to type IV collagen (Mayer et al., 1991) and to other collagens (Sage et al., 1989; Lane and Sage, 1990), and that binding of Ca^{2+} to this motif elicits a major conformational change in the protein (Engel et al., 1987). A second, more ambiguous EF-hand motif may also be present in domain III of chicken SPARC. The identification of this second motif is based on revised consensus patterns in which all published sequences containing EF-hands have been taken into account (Bairoch, 1992). Bovine SPARC has also been described as having two EF-hands (Bolander et al., 1988). However, this site is not conserved in mouse (Engel et al., 1987) or frog (Damjanovski et al., 1992) and might not be functionally significant.

SPARC also binds Cu^{2+} (Vernon and Sage, 1989; Lane, T. F., Iruela-Arispe, M. L., Johnson, R. and Sage, E. H., unpublished results). The importance of this observation is highlighted by prior studies that implicate Cu^{2+} in the modu-

lation of neovascularization (Brem et al., 1990). To address which amino acids are involved in the binding of Cu^{2+} by SPARC, we considered the consensus signatures of the following known Cu^{2+}-binding proteins: (a) type I or blue-copper proteins, (b) type II ascorbate-dependent monooxygenases, (c) copper/zinc superoxide dismutases, (d) cytochrome oxidases and (e) ceruloplasmin. None of these signatures was found within chicken SPARC. We did observe, however, that domain 2.3 of SPARC contains the Cu^{2+}-binding tripeptide, GHK, which is known to induce the proliferation of many cell types (Pickart and Lovejoy, 1987) and the expression of collagen by fibroblasts (Maquart et al., 1988). This tripeptide thus represents the likely site of Cu^{2+} chelation by SPARC. This GHK sequence is contained within a conserved KKGHK sequence that is found in only three proteins. Interestingly, peptides (18 amino acids) derived from this region of SPARC also bind Cu^{2+} (Lane, T. F., Iruela-Arispe, M. L., Johnson, R. and Sage, E. H., unpublished results).

We evaluated the hydrophilicity and hydrophobicity of chicken SPARC along its amino acid sequence through use of a hydropathy scale (Kyte and Doolittle, 1982), in which the hydrophilic and hydrophobic properties of each of the 20 amino acid side chains are taken into consideration. Chicken SPARC contains eight-residue and nine-residue hydrophobic regions which are likely to comprise the interior core of the protein. Punctuated throughout the protein are smaller clusters of hydrophobic residues that could interact with each other during folding. The putative Ca^{2+}-binding EF-hand found in domain III (Asp160–Lys171) would then be present within a large hydrophilic loop (Thr155–Thr190). The C-terminus of this hydrophilic loop is consistent with the major site of proteolysis observed during the purification of SPARC (Mann et al., 1987; Maurer et al., 1992).

A 41–43-kDa protein from chicken heart/aorta, lung, limb and calvarium was detected with anti-SPARC immunoglobulins. The presence of SPARC in the developing chicken vasculature is consistent with roles proposed for the protein in angiogenesis (Iruela-Arispe et al., 1991), whereas its presence in limb and calvarium probably represents synthesis concurrently with osteogenesis (Termine et al., 1981; Leboy et al., 1988) and chondrogenesis (Oshima et al., 1989; Pacifici et al., 1990). SPARC was also present in abundant quantities in the conditioned media of chicken smooth muscle cells, as previously noted for cultured chicken chondrocytes (Neri et al., 1992). We have previously observed the expression of SPARC by muscle tissue *in vivo*. For example, SPARC protein has been immunologically detected in skeletal muscle derived from tongue and diaphragm (Sage et al., 1989b) and in myoblasts (Sage et al., 1992). The appearance of SPARC in developing muscle provides further evidence for its possible function as a modulator of cell growth and differentiation.

An intriguing result was the detection of the 1.8-kb and 2.2-kb SPARC RNAs in the brain of 10-day-old chicken embryos. The presence of the 2.2-kb SPARC RNA in the adult rat brain has been noted previously (Johnston et al., 1990), whereas the detection of SPARC RNA in the embryonic brain is a novel finding. We were therefore surprised that our anti-SPARC immunoglobulins recognized proteins of 25 kDa and 110 kDa and not the expected 41–43-kDa SPARC. These proteins appear to be related to SPARC, since incubation of anti-SPARC immunoglobulins with SPARC peptide 4.2 blocked the immunoreactivity. Since peptide 4.2 contains the EF-hand sequence, it is indeed likely that the 25-kDa and 110-kDa proteins might also contain this Ca^{2+}-binding element.

We considered four possibilities to explain our detection of the 3.0-kb and 1.8-kb RNAs with the chicken SPARC cDNA probe: (a) the RNA transcripts were derived from genes other than SPARC, (b) differential RNA processing occurred at the intron/exon level, (c) differential RNA processing occurred from an alternative poly(A) site and (d) a pseudogene exists which gave rise to the additional RNA species. The 3.0-kb RNA might represent the chicken analog of rat brain SC1. Although SC1 RNA has been detected throughout postnatal development of the rat brain and in the adult (Johnston et al., 1990), we could not detect the 3.0-kb SPARC RNA in 10–day-old chicken embryonic brain. The relative steady-state levels for the 2.2-kb and 1.8-kb RNAs are in agreement with our prior localization of SPARC protein and RNA in tissues and organs of the mouse (Holland et al., 1987; Sage et al., 1989b). The high levels of the 2.2-kb RNA in myoblasts (Holland et al., 1987; Sage et al., 1992) have been noted previously and might reflect a role for SPARC in a specific stage of muscle differentiation, since expression in skeletal muscle is not observed in the adult. The primary transcripts of SPARC are known to have two or more alternative poly(A) sites (Swaroop et al., 1988). Sequence analysis of the major 2.2-kb and minor 3.0-kb human mRNAs indicated that both transcripts were produced from a single gene and were generated by the differential utilization of transcriptional termination signal sequences (Swaroop et al., 1988). Thus, the 3.0-kb chicken RNA observed in this study could have arisen from the failure of RNA polymerase to terminate transcription at the major termination site (ATAAA) in the chicken SPARC gene. We have not recognized any potential polyadenylation signal sequences which could generate the 1.8-kb RNA present in chicken tissues and organs. Although our data presently do not support alternative splicing of primary SPARC transcripts, we cannot exclude this level of RNA processing. Since total RNA was used in our Northern-blot analyses, the three bands we observed may not be represented in cytoplasmic mRNA. Our recent acquisition of genomic clones encoding chicken embryo SPARC will now allow us to examine directly the regulation of expression of these RNA transcripts in different tissues.

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