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The hinge region of chicken annexin I contains no site for tyrosine phosphorylation

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Annexin I (AnxI) is a calcium-dependent membrane binding protein which has been implicated in various physiological activities. The region of the chicken *anxI* cDNA encoding the first 130 amino terminal residues was cloned by reverse transcription PCR in order to determine the relationship of its variable amino-terminal regulatory region with other known annexins. This nucleotide sequence shows 86% identity with pigeon AnxI isoforms, and 57% with its human homolog. The protein encoded by the chicken *anxI* cDNA lacks the canonical epidermal growth factor receptor/kinase phosphorylation site, which is present in AnxI of other species In contrast, the putative protein kinase C phosphorylation site of the amino-terminus is present in the chicken AnxI. Whereas the pigeon genome contains two *anxI* genes, genomic Southern analysis shows that in the chicken AnxI is encoded by only a single gene. These data suggest that AnxI has undergone significant sequence variation in the avians, and clarifies the relationships of the avian *anxI* genes with their ancestral homologs.

Annexin I; Lipocortin I; Tyrosine kinases; Protein kinase C; Molecular evolution; Chicken

1. INTRODUCTION

Annexin I (AnxI; also referred to as lipocortin I) is a member of a class of homologous proteins that bind to membrane phospholipids in a calcium-dependent manner. There are at least 12 distinct members in this group, which are widely distributed across both species and tissues and have been implicated in diverse physiological activities (see [1–3] for reviews). While both the Ca^{2+} and the membrane binding activities of the annexins reside in the core domain, the amino-terminal tail plays a role in the regulation of these proteins [4–8].

AnxI is one of the best characterized members of the group [9,10]. While mammals have only one *anxI* gene encoding a single protein product, the pigeon genome contains two *anxI*-type genes. The first to be discovered, *cp35*, is a major prolactin-induced gene in the cropsac. Its protein product is tissue-specific and lacks the typical AnxI phosphorylation sites in its leader domain [11]. The second isoform, referred to as *cp37*, encodes a protein which is constituively expressed, and contains AnxI consensus phosphorylation sites [12]. The two pigeon isoforms share 93% amino acid identity, but differ considerably in the hinge region, which is encoded by the first half of exon three [10]. Based upon these findings it was speculated that the phosphorylation sites of cp37 evolved independently in the Aves.

We now report the cDNA sequence of a large amino-terminal portion of chicken AnxI and present evidence that this gene exists as a single copy in the chicken. The chicken sequence clarifies the evolution of *anxI* in birds, and provides new insights into the regulation of its protein.

2. EXPERIMENTAL

2.1. *Northern blot assay of annexin I mRNA*

Total RNA from the cropsac of prolactin treated (200 μg/day for 3 days) pigeons and from liver, lung and cropsacs of 1- or 3-day-old chickens, were extracted by RNAzol B (Cinna/Biotecx, Houston, TX, USA) and electrophoreses (15 μg/lane) through 1 1% agarose, 1.1 M formaldehyde gels containing 0.5 μg/ml ethidium bromide After electrophoresis the samples were transferred to nylon membranes (Nytran, Schleicher & Schuell, Keene, NH) by capillary blotting and probed by standard Northern blot technique [12].

2.2. *Genomic DNA extraction and Southern analysis*

Chicken liver tissue fragments (~0.5 g) were ground in liquid nitrogen and digested in 6 ml of digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8, 25 mM EDTA, pH 8, 0.5% SDS, 20 μg/ml RNAase and 0.1 mg/ml proteinase K) at 50°C for 16 h. The DNA was then extracted 3 times with an equal volume of phenol/chloroform/isoamyl alcohol, precipitated with 1/2 vol. of 7.5 M ammonium acetate and 2 vol. of 95% ethanol and recovered as described [13]. Genomic DNA aliquots were restriction digested, ethanol precipitated, and fractionated (20 μg/lane) on 0.8% agarose gel The DNA was then transferred to a nylon membrane, fixed and hybridized [14]. For reprobing, the blots were stripped by boiling for 20 min in 0.1 × SSC and 0.5% SDS.

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Abbreviations EGF, epidermal growth factor; PKC, protein kinase C; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; AnxI, annexin I.

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2.3. *Oligonucleotide primers*

Oligonucleotides used in polymerase chain reaction (PCR) were synthesized according to previously reported AnxI sequences of sponge, human and pigeon ([15,16] and [11] and [12], respectively). The sense primers (5'-CGCGCATGCATGGCTATGGTATCAGAATTTC-3') corresponded to the characteristic conservative segment at the beginning of AnxI type sequences and designed to contain a *Sph*I restriction site (underlined) and CGC clamp at the 5' end. The anti-sense primer (3'-CCCGAACCTTGACTACTTCTACTCGAGCGC-5') contained a *Sac*I site (underlined) and corresponded to a conservative sequence located at the end of exon 5 and the beginning of exon 6 (see the primer positions in Fig 3).

2.4 *PCR amplification of chicken AnxI cDNA*

Single-stranded cDNA was synthesized in a 20 μl reaction containing: 1 × Taq DNA Polymerase buffer, 1.25 mM $MgCl_2$, 0.1–0.2 μg total RNA, 5 pmol antisense primer, 20 U of RNasin ribonuclease inhibitor (Promega), 1 mM of each deoxynucleotide triphospate (dNTP) and 7.5 U of AMV Reverse Transcriptase (Promega). The mixture was incubated at 42°C for 1 h and the reaction terminated by heating at 95°C for 5 min. The reverse transcription reaction was then diluted to a final volume of 100 μl in 1 × Taq DNA polymerase buffer or Pfu DNA polymerase buffer, 5 μM of each of the sense and antisense primers and 5 U of either Taq or Pfu DNA polymerase. Amplification was performed at a temperature profile of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, for 38 cycles. The right size product was extracted from a 1% agarose gel using a Prep-A-Gene DNA purification kit (Bio-Rad Lab. Richmond, CA). Reamplification reactions were carried out by the same procedure as the above amplification reaction using 1 ng of the purified cDNA fragment as a template

2.5. *Cloning and sequencing the PCR cDNA fragment*

The double-strand PCR products were digested with *Pst*I and *Sac*I, purified and ligated into cleaved and dephosphorylated pGEM3 plasmids (Promega). Competent cells (DH5a, BRL) were transformed and the positive colonies were selected by ampicilin resistance Five independently cloned PCR products were sequenced on both strains, by the dideoxy chain termination method using Sequenase (US Biochemical, Cleveland, OH).

2.6. *Sequence alignment*

The degree of identity between chicken nucleotides and amino acid sequences and other AnxI relatives, was calculated for the various structural domains of the protein, from optimal pairwise alignments. For these calculations the sequences defined by the PCR primers were ignored. This includes: the Leader Domain – residues 8–20; the Hinge Region – residues 21–41 (human AnxI numbering); and residues 42–128 of the Core Domain. The alignments were obtained independently for each pair, by CLUSTAL V software [17].

3. RESULTS AND DISCUSSION

Northern blot analysis of chicken lung, liver and cropsac revealed high level expression of a 1.4 kb mRNA in the cropsac, but not in liver or lung (Fig. 1). Cropsac RNA was used for reverse transcription PCR (rtPCR) of the desired AnxI fragment. The 410 bp PCR product, proven by sequencing to be AnxI (see below), was used as a probe in genomic Southern blot analysis (Fig. 2A). The various restriction enzymes generated 1–5 hybridizing bands which ranged between 0.5–8 kb. This contrasts with *anxI* in pigeon genome, which yielded a much more complex Southern hybridization pattern [14]. The hybridization patterns of chicken *anxI* and *vimentin* (Fig. 2B) were of similar complexity. Vimentin is encoded by a single gene in the chicken (9 exons, 8.5 kb; [18]). These data indicate that chicken *anxI*, unlike pigeon, is a single-copy gene.

Chicken *anxI* cDNA, synthesized independently by both Taq and Pfu DNA polymerases, was cloned and sequenced. The resulting 390 base sequence obtained from 5 independent clones, is presented in Fig. 3, along with its deduced polypeptide of 130 amino acids. The 130 amino acid region that we cloned for the chicken corresponded to the entire amino-terminal regulatory domain, all of repeat 1 and the first helix of repeat 2. This fragment was chosen because the intent was primarily to analyze the variable amino-terminus, not to repeat the cloning of the conserved core domain. As would be expected in pairwise alignments of the 130 amino acid region, the chicken polypeptide sequence shared higher identity with both pigeon relatives (80% on average) than with either human (72%) or sponge (55%).

It is well established that Tyr-21 of the hinge region of mammalian AnxI is a high affinity substrate for the EGF receptor/kinase [19,20]. Phosphorylation of Try-21 renders AnxI 20-fold more sensitive to proteolysis at Lys-26 [20]. The hinge region also contains substrate sites for PKC at Ser-27 and Thr-24 [7]. The chicken AnxI like pigeon cp35, has a cysteine in position 21, instead of the tyrosine contained by all other relatives (Figs. 3 and 5). With cp37 it shares the Ile-Lys-Ser PKC

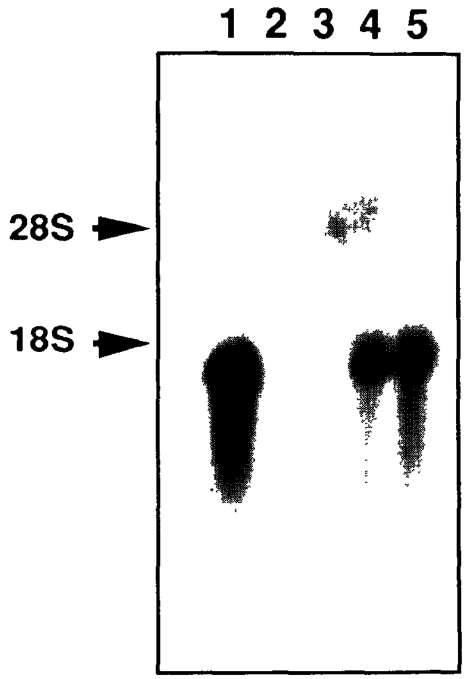

Fig. 1. Expression of AnxI in chicken tissues. Total RNA from prolactin-stimulated pigeon cropsac (lane 1 = positive control) and from 1-day-old chicken liver (lane 2), lung (lane 3) and cropsac (lanes 4 and 5) were separated through a 1% formaldehyde-containing agarose. The RNA was then capillary transferred to a nylon membrane and probed with nick-translated cp35 insert cDNA.

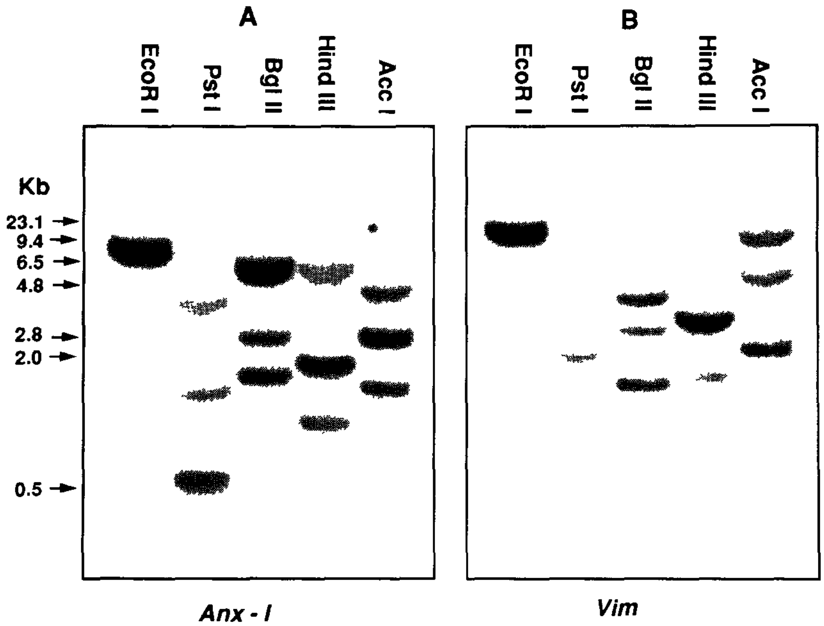

Fig 2. Genomic Southern analysis of chicken AnxI. High molecular weight chicken liver DNA was digested with restriction enzymes (as noted above the figure) and fractionated (20 μg/lane) on 0.8% agarose gel. Following capillary transfer the blot was probed with chAnxI cDNA fragment (A), stripped and reprobed with nick-translated pigeon vimentin cDNA insert (B).

phosphorylation site at positions 22–24, and the Lys-Gly-Gly motif at positions 26–28 (Figs. 3 and 5). The following 6 amino acids (29–34) show almost no identity with either pigeon isoform nor with nonavian relatives (see alignment in Fig. 5).

A multiple alignment of the hinge region nucleotide sequences from all species studied to date is shown in Fig. 4. Gaps in the alignment of avian AnxI and other species were located at two separate locations: positions 77–84 (numbered from the initiation codon) and 98–100. Except for a short sequence of 8 nucleotides (positions 104–111), most of the hinge region showed a re-

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            *            *            *             *            *
ATG GCT ATG GTA TCA GAA TTT CTG AAG CAG GCA TGG TTC ATG GAC AAC CAG  51
Met Ala Met Val Ser Glu Phe Thr Lys Gln Ala Trp Phe Met Asp Asn Gln  17

          *             *            *            *             *
GAG CAG GAA TGT ATT AAA AGT TCA AAA GGT GGC TCT TCA GTA CAG TCA CGC 102
Glu Gln Glu Cys Ile Lys Ser Ser Lys Gly Gly Ser Ser Val Gln Ser Arg  34

         *            *             *            *            *
CCA AAC TTT GAT CCC TCA GCT GAT GTT TCT GCT TTG GAT AAA GCT ATT ACT 153
Pro Asn Phe Asp Pro Ser Ala Asp Val Ser Ala Leu Asp Lys Ala Ile Thr  51

        *            *            *             *            *
GTA AAG GGT GTA GAT GAA GCC ACC ATC ATT GAC ATC TTG ACT AAA AGA ACA 204
Val Lys Gly Val Asp Glu Ala Thr Ile Ile Asp Ile Leu Thr Lys Arg Thr  68

      *             *            *            *             *
AAT GCT CAA CGT CAG CAG ATC AAA GCT GCC TAT CAA CAG GCA AAA GGA AAG 255
Asn Ala Gln Arg Gln Gln Ile Lys Ala Ala Tyr Gln Gln Ala Lys Gly Lys  85

     *            *             *            *            *
AGC CTG GAA GAA GAC TTG AAA AAG GTG CTG AAA AGC CAC TTG GAA GAT GTT 306
Ser Thr Glu Glu Asp Leu Lys Lys Val Thr Lys Ser His Leu Glu Asp Val 102

    *            *            *             *            *
GTT GTT GCT CTT CTC AAA ACT CCA GCT CAG TTT GAT GCT GAA GAA TTA AGG 357
Val Val Ala Thr Thr Lys Thr Pro Ala Gln Phe Asp Ala Glu Glu Leu Arg 119

  *             *            *            *
GCC TCT ATG AAG GGG CTT GGA ACT GAT AGA AGA 390
Ala Ser Met Lys Gly Thr Gly Thr Asp Arg Arg 130
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Fig. 3. Nucleotide and deduced amino acid sequences of chAnxI cDNA fragment. Chicken cropsac total RNA (0.1 μg) was reverse transcribed and amplified by PCR using primers which are specific for AnxI (underlined). The PCR products were cloned and sequenced as described.

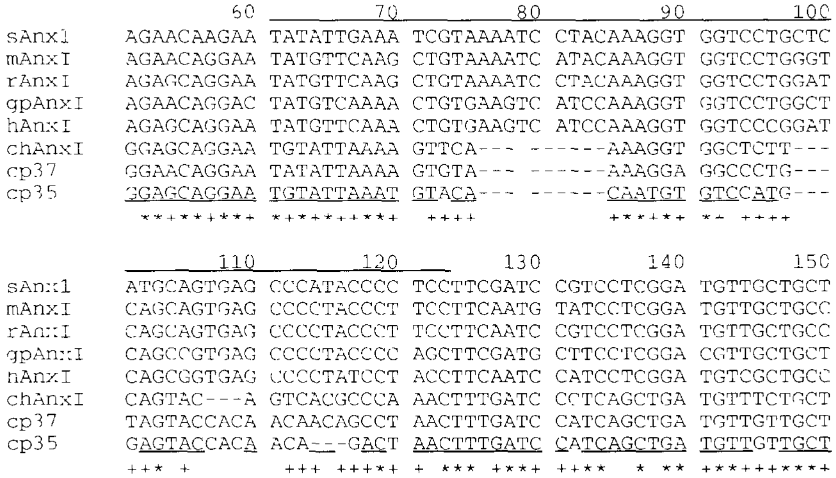

Fig. 4. Multiple alignment of nucleotide sequences of the hinge region (indicated by the upper line) of AnxI from sponge (sAnxI; [15]), mouse (mAnxI; [21]), rat (rAnxI; [22]), guinea pig (gpAnxI; [23]), human (hAnxI; [16]), chicken (chAnxI; this paper) and pigeon (cp35 and cp37; [11] and [12], respectively). Gaps were automatically introduced by CLUSTAL V software. The asterisk and plus symbols on the bottom of the sequence indicate similarity between avian and nonavian sequences. Asterisks represent positions that are identical in all sequences, plusses indicate positions in which the same base appears in at least one of the avian and one of the nonavian sequences. Identical bases shared by chicken and at least one of the pigeon isoforms are underlined.

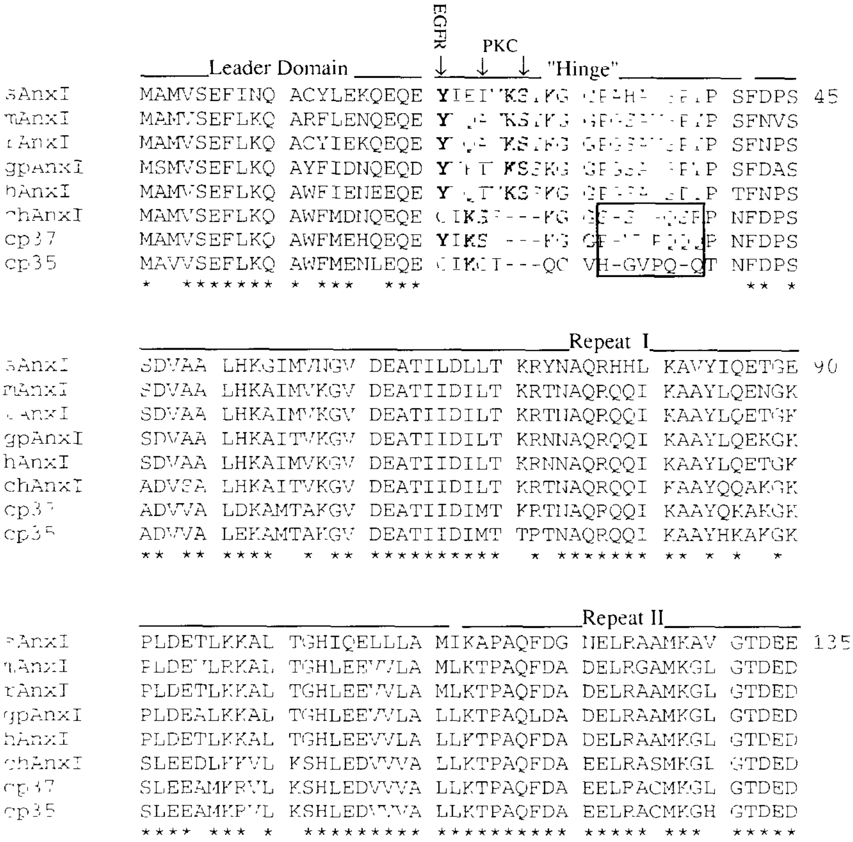

Fig. 5. Comparison of the amino acid sequences of the leader domain (residues 1–20) the hinge region (residues 21–41) and the first two repeats of the core domain (residue 43–135) of AnxI. Species are as listed in Fig. 4. Gaps were inserted according to the nucleotide sequence alignment rather than by independent alignment of the amino acid sequence. Identical and conservative substitutions across the sequences are indicated by asterisks. The arrows denote the phosphorylation sites for PKC and for the EGF receptor/kinase in mammals. Motifs which are conserved between avians (excluding cp35) and nonavians are shadowed. A segment that is highly diverse within the avians is boxed.

markably high similarity between avian and nonavian sequences (Fig 4). Therefore, it seems reasonable to conclude that in spite of the low amino acid identity, the avian hinge region is homologous to the nonavian one. Its diversity then, is the result of deletion events and base substitutions, rather than an insertion of a new segment of DNA [12].

The amino acid sequence of the chicken AnxI fragment and other homologues are compared in Fig. 5. In the leader domain and the first repeat segments the avian sequences showed a great deal of similarity with the nonavian, with most of the substitutions being conservative. While the selective pressure acting to conserve the structure of the core domain is evident, the reason for strong conservation in the leader domain is still to be discovered. In the hinge region the similarity between avians and nonavians was found to be very low (Fig. 5). In conformity with the nucleotide alignments (Fig. 4) 3- and 1-residue gaps were placed at positions 26 and 33, respectively. An alternative alignment in which all missing residues were grouped together in a single gap at positions 35 for cp35, and 36 for cp37 and chAnxI, was obtained by CLUSTAL V [12]. However, the two-gap alignment, consistent with the nucleotide analysis, was preferred since it offers better interpretations of the homologous relationships between sequences. The alignment in Fig. 5 suggests that the Lys-Ser, putative PKC phosphorylation site, shared by chAnxI and cp37, are encoded by codons which are not homologous with those encoding the analogous residues in nonavians. Furthermore, Lys-26 (per human numbering), which is a protease-sensitive site, is also not homologous between the two groups. It is of interest to note that in the avian sequences, Lys-29 (per human numbering) together with the successive Gly-Gly residues are homologous with identical sequences, in other species. Another feature of avian AnxI is a highly variable segment corresponding to Ser-29 through Arg-34 of chAnxI. At the polypeptide sequence level the changes outlined here appear complex, but they require a minimal number of nucleotide changes (Fig. 4).

Based on the above observations one can propose that the hinge region of avian *anxI* genes arose by modifications of the ancestral gene sequences that preserved some of the potential regulatory mechanisms, but eliminated others. The presence of the two AnxI genes in columbids is a special case. Our model suggests that the PKC Ser-24 site is the major regulation site in AnxI, and that Tyr-21 plays a subsidiary role. The polymorphism of AnxI genes in the two avian species examined to date argues that an extensive analysis of avian AnxI will yield important molecular and evolutionary insights.

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