Primary Structure of the Brain $\alpha$-Spectrin

Veli-Matti Wasenius,*, Matti Saraste,* Petri Salvén,* Marja Erämaa,* Liisa Holm,§ Veli-Pekka Lehtoll†

*Department of Pathology and †Department of Medical Chemistry, University of Helsinki, Helsinki, Finland; §Biotechnical Laboratory, Technical Research Center of Finland, Espoo, Finland; and †Biocenter and Department of Pathology, University of Oulu, Oulu, Finland

Abstract. We have determined the nucleotide sequence coding for the chicken brain $\alpha$-spectrin. It is derived both from the cDNA and genomic sequences, comprises the entire coding frame, 5' and 3' untranslated sequences, and terminates in the poly(A)-tail. The deduced amino acid sequence was used to map the domain structure of the protein. The $\alpha$-chain of brain spectrin contains 22 segments of which 20 correspond to the repeat of the human erythrocyte spectrin (Speicher, D. W., and V. T. Marchesi. 1984. Nature (Lond.). 311:177-180.), typically made of 106 residues. These homologous segments probably account for the flexible, rod-like structure of spectrin. Secondary structure prediction suggests predominantly $\alpha$-helical structure for the entire chain.

Parts of the primary structure are excluded from the repetitive pattern and they reside in the middle part of the sequence and in its COOH terminus. Search for homology in other proteins showed the presence of the following distinct structures in these nonrepetitive regions: (a) the COOH-terminal part of the molecule that shows homology with $\alpha$-actinin, (b) two typical EF-hand (i.e., Ca$^{2+}$-binding) structures in this region, (c) a sequence close to the EF-hand that fulfills the criteria for a calmodulin-binding site, and (d) a domain in the middle of the sequence that is homologous to a NH$_2$-terminal segment of several src-tyrosine kinases and to a domain of phospholipase C. These regions are good candidates to carry some established as well as some yet unestablished functions of spectrin. Comparative analysis showed that $\alpha$-spectrin is well conserved across the species boundaries from Xenopus to man, and that the human erythrocyte $\alpha$-spectrin is divergent from the other spectrins.

Spectrin is the major constituent of the cytoskeletal network underlying the plasma membrane (for a review see Marchesi, 1985). It was considered to be specific for red blood cells until spectrin-like proteins were detected immunologically in many types of cells (Goodman et al., 1981; Levine and Willard, 1981; Bennett et al., 1982; Burridge et al., 1982; Glenney et al., 1982a,b; Repasky et al., 1982; Kakiuchi et al., 1982; Lehto and Virtanen, 1983). At present we know several proteins related to the red blood cell spectrin. Their kinship has been primarily investigated by peptide mapping and immunological techniques (Repasky et al., 1982; Glenney et al., 1983; Glenney and Glenny, 1984a, b; Harris et al., 1985). These studies have shown that spectrins in different tissues occur as heterodimers and possess a common ( $\alpha$, $M$, from 230 to 260 kD) and a variant ( $\beta$ or $\gamma$, $M$, from 220 to 260 kD) subunit (Lazarides and Nelson, 1985). The latter show a high degree of variation while the common subunits are much alike in different types of cells (Glenney et al., 1982b). The mammalian erythroid $\alpha$-chain is, however, a deviant member of the family and diverges from the others by its immunological and structural properties (Glenney and Glenny, 1984a; Harris et al., 1985).

The first spectrin-like molecule to be detected outside the realm of the red blood cells was found in brain (Levine and Willard, 1981; Goodman et al., 1981) and is also called fodrin (Levine and Willard, 1981) or calspectin (Kakiuchi et al., 1982). Recently it has been shown that mammalian brain contains two isoforms of spectrin (Lazarides and Nelson, 1983; Lazarides et al., 1984; Riederer et al., 1986; Virtanen et al., 1986), one located primarily in the axons and the other in the cell bodies and dendrites. Another spectrin-like protein that is more thoroughly characterized, was detected in the avian intestinal tissue (Glenney et al., 1982b). This terminal web (TW) 260/240-protein differs from the others by its location in the terminal web of the enterocytes, distant from the plasma membrane.

First data on the primary structure of spectrin were presented by Speicher et al. (1983). They sequenced some tryptic peptides of the human erythrocyte spectrin $\alpha$- and $\beta$-chains. The sequences revealed repetitive structure where each unit typically consists of 106 amino acids. However, the compiled fragmentary data cover only $\sim$48% of the $\alpha$- and 18% of the $\beta$-chain (Speicher and Marchesi, 1984). The first cDNA sequence of a nonerythroid spectrin was determined by us for a clone isolated from a chicken gizzard expression library (Wasenius et al., 1985). This 1.5-kb sequence showed that the basic architecture of the erythrocyte spectrin (i.e., the multiply repeated 106-residue motif) is also found in the nonerythroid spectrins. This finding was confirmed by Birkenmeier et al. (1985). Later, McMahon et al. (1987), Leto et al. (1988), and Giebelhaus et al. (1987) have provided...
partial sequences of human spectrin, rat and human spectrins, and of Xenopus oocyte spectrin, respectively.

In this paper we present the primary structure of α-spectrin, based on the cDNA and genomic sequences, and map its domain structure. The primary structure reveals a multi-domain molecule. It is mostly comprised of regular homologous repeats but also contains in its middle and COOH-terminal parts nonrepetitive sequences. The latter show sequence similarities to some functionally defined proteins and may thus carry specific functions of spectrin.

**Materials and Methods**

**General Methods and Reagents**

General procedures, such as isolation of DNA, restriction enzyme digests, PAGE and agarose gel electrophoresis, purification of DNA fragments from the gels, and nick translation of the probes, were performed by standard methods (Maniatis et al., 1982). Restriction and modification enzymes and polymerases were purchased from Boehringer Mannheim Biochemical GmbH (Mannheim, FRG) unless indicated otherwise and radiolabeling was done using [α-32P]dCTP (Amersham International, Amersham, UK).

**Isolation of cDNA and Genomic Clones**

A cDNA library was made in lambda g10 phage as described (Huynh et al., 1985). Total RNAs were prepared from various tissues of 14-d chicken embryos by a modified guanidine isothiocyanate/cesium chloride method as described earlier (Chirgwin et al., 1979). Poly (A)+-RNA was isolated by two cycles on an oligo-dT-cellulose column (Pharmacia Fine Chemicals, Piscataway, NJ). The RNA isolated from brain was used for cDNA synthesis. This was carried out by K.Nase H method (Gubler and Hoffman, 1983) using oligo-dT (Promega Biotec, Madison, WI) as a primer. cDNA was then treated with Eco RI - methylase, Eco RI linker digest, digested with Eco RI, and size selected on a Sepharose CL-4B column (Pharmacia Fine Chemicals). cDNA fragments over 1 kb were ligated overnight at 14°C to the Eco RI-digested and phosphatase-treated lambda g10 vector (Promega Biotec) and packaged into virus particles. Escherichia coli c600hn host cells were transformed and plated. A chicken genomic library was constructed by using the DNA isolated from the brains of 14-d-old chicken embryos. The DNA was partially digested with Eco RI, ligated into lambda g10-vector, packaged, and transfected into E. coli c600hn cells following the procedures described for cDNA library construction (see above).

The cDNA library was spread on culture plates, replicated onto nitrocellulose filters which were then screened by hybridization with nick-translated 18-3a clone (Wasenius et al., 1985). Screening of ~1.8 × 10⁶ plaques under stringent conditions (final wash with 0.1x SSC, 0.1% SDS at 65°C for 1 h) yielded 23 positive clones. The positive clones were localized, picked up, and plaque purified. The isolated lambda DNAs were digested with Eco RI to release the inserts. These fragments were isolated from agarose gels by trapping to strips of DEAE-nitrocellulose filters and subcloned into M13 vectors that would extend the sequence further into the 5' direction. Thus a 17-mer primer (5'CTCTTCCAGAAGATTCT3') was used to prime a sublibrary which was screened with the CLONE IA clone. The DNA was partially digested with Eco RI, ligated into lambda g10-vector, packaged, and transfected into E. coli c600hn cells following the procedures described for cDNA library construction (see above).

**DNA Sequence Analysis**

Sequencing of the DNAs subcloned into M13 mpl8 and mpl9 vectors was performed by Sanger's dyeoxy chain termination method (Sanger et al., 1980; Biggin et al., 1983) with both the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, MD) and modified T7 DNA polymerase from United States Biochemical Corp. (Cleveland, OH). Exo III-nuclease technique was used to generate deleted inserts (Henikoff, 1984). M13 universal primer and several specific primers were used.

**Computer Analysis**

Nucleotide sequencing was aided by the Staden programs (Staden, 1987). Amino acid sequence homologies were studied with a computer program, DIAGON (Staden, 1982); the parameters in dot matrix analyses are specified in the figure legends. Secondary structure was predicted using the program of Garnier et al. (1978). Search for homologous sequences was carried out using the protein identification resource, PIR (database 12.0, March 1987), as a reference.

**Oligonucleotide Synthesis**

Decoy oligonucleotides were synthesized with synthesizer (model 381A; Applied Biosystems, Inc., Foster City, CA).

**Results**

**Isolation of cDNA and Genomic Clones for Brain α-Spectrin**

Brain was chosen for the construction of the cDNA library on the basis of its relatively high content of spectrin mRNA; this was found by Northern hybridization of mRNAs from various tissues with the α-spectrin probe 18-3a (not shown). The library was screened by hybridization with the same probe. The longest cDNA insert detected was ~4.7 kb. The nucleotide sequence analysis revealed that its 3' end corresponded to the poly(A)+-tail of the mRNA. The clone contains an authentic Eco RI site that divides it into two halves which are designated CLONE IA and CLONE IB in Fig. 1. The 18-3a sequence is present in the middle of the CLONE I, confirming that the isolated clone represents α-spectrin; it is marked with a dashed underline in Fig. 2 (nucleotides 5,041-6,459). Sequencing errors in the 5' end of the published 18-3a sequence (Wasenius et al., 1985) and in the 12 carboxy-terminal residues in our previously published partial sequence (Wasenius et al., 1987) are corrected in Fig. 2.

We were unable to isolate from the original cDNA library clones that would extend the sequence further into the 5' direction. Thus a 17-mer primer (5'CTCTTCCAGAAGATTCT3') was used to prime a sublibrary which was screened with the CLONE IA clone. The DNA was partially digested with Eco RI, ligated into lambda g10-vector, packaged, and transfected into E. coli c600hn cells following the procedures described for cDNA library construction (see above).

**Figure 1. Restriction enzyme map and sequencing strategy for the α-spectrin cDNA and genomic clones.** The mapped restriction sites are Bam HI (B), Eco RI (E), Hind III (H), Pst I (P), and Sph I (S). The cDNA sequence was determined from both ends using the Eco RI and Hind III sites. The genomic clone CLONE I, that contains the 5' end of the coding sequence, was isolated using the primer CLONE IA and primer A. The genomic clone CLONE II, that contains the 5' end of the coding sequence, was isolated using the primer CLONE IA and primer A. The genomic clone CLONE III, that contains the 5' end of the coding sequence, was isolated using the primer CLONE IA and primer A. The genomic clone CLONE IV, that contains the 5' end of the coding sequence, was isolated using the primer CLONE IA and primer A.
was designed complementary to the 5′ end of the CLONE IB (Fig. 1) and used as a primer to produce a sublibrary using the RNase H method described in Materials and Methods. The sublibrary was screened with the CLONE IB as a probe. From it a new clone (CLONE II) that overlaps the original 4.7-kb clone was obtained.

To obtain clones covering the 5′ end of the coding sequence further, the genomic library was screened with some 5′ fragments of the CLONE II as probes. This yielded a ~7-kb genomic clone (CLONE G/I) that overlaps the CLONE II by ~650 bp.

The restriction map and the sequencing strategy of the CLONES I, II, and G/I are summarized in Fig. 1.

**The Nucleotide and Deduced Amino Acid Sequence of the Brain α-Spectrin**

The nucleotide sequence of the entire coding region was obtained from the two overlapping cDNA clones and from a partial sequence of the genomic clone. Also, the entire untranslated 3′ region was sequenced. Fig. 2 shows the consensus nucleotide and deduced amino acid sequences. The former comprises 7,774 bp and contains an open reading frame (7,431 nucleotides) that codes for 2,477 amino acid residues yielding a mol mass of 285,369 for the translation product. The putative initiation codon is 124 bases from the 5′ end of the shown sequence. It is flanked by a sequence that is in good agreement with the Kozak’s rule for the functional initiation codon (Kozak, 1986). This ATG codon is preceded by a typical TATA box (between −45 and −40) and a CAAT box (−111 to −108). After the stop codon, there is a 219-bp untranslated 3′ sequence with a poly(A)-tail. The polyadenylation signal AAATAA occurs 19 bp from the poly(A)-tail (nucleotides 7,606–7,611). These signals are underlined in Fig. 2. The Eco RI site in CLONE I was not overlapped by sequencing. However, a previously determined homologous sequence has already covered this site (McMahon et al., 1987).

**Repetitive Structure of the α-Spectrin**

Internal repeats were systematically studied using a computer program, DIAGON (Staden, 1982). Fig. 3, depicting a diagonal plot, shows that the molecule is composed of homologous NH2- and COOH-terminal halves indicated by a long contiguous diagonal line (Fig. 3, arrow), and of multiply repeated, homologous units indicated by the numerous parallel evenly spaced lines. These basic repeats are designated α1, α2, α3, etc. following the nomenclature introduced by Speicher and Marchesi (1984). They encompass ~80% of the molecule excluding two regions (α10 and α11) in the middle and one (α21 and α22) at the carboxy-terminal part of the protein (Fig. 4). The latter are revealed as “white strips” in the dot matrix, which indicates that they are different from the other segments.

The optimal alignment of the homologous repeats is shown in Fig. 4. The particular alignment (α1 begins with the amino acid residue 15) has been chosen to make it match with the published pattern of Speicher and Marchesi (1984) and of Wasenius et al. (1985). This leaves 14 NH2-terminal residues as an overhang, designated α1. Most of the repeats (α2–5, α7–9, α12–14, and α16–18) are 106 amino acids long, thus conforming to the common repetitive pattern of the spectrin structure (Speicher and Marchesi, 1984; Wasenius et al. 1985). One gap is required for the optimal alignment in α1 and α6. α15 is longer than the other repeats due to the unique insertion in position 70 (Fig. 4).

The alignment shows a faithful occurrence of certain amino acids in the fixed positions in most of the repeats (Fig. 4). These include isoleucine (in positions 1 and 46), tryptophan (12 and 45), leucine (15 and 26), arginine (22), aspartic acid (38), glutamic acid (48), lysine (71), and histidine (72 and 101). The amino-terminal ends of the repeats seem to be more strongly conserved than the carboxy-terminal ends.

The segments α20 and α21 are longer and show a lower degree of homology to the other repeats. In addition, the segments called α10 and α22 are qualitatively different from the rest of α-spectrin chain. Another “nonhomologous” region can be found as an extension of the α11 unit.

In Table I all the pairwise comparisons of the units α1–α22 are shown. The highest degree of homology is seen between the repeats in the corresponding positions of the amino- and carboxy-terminal halves of the molecule; the α2–α8 stretch is closely related to a sequence covering α11–α17 (Fig. 3, arrow). This may be a track left by the latest duplication event in the evolution of spectrin (see Discussion). This comparison also shows that the divergent regions α10 and α22 are totally unrelated to the homologous repeat units and to each other.

Secondary structure prediction was carried out using the algorithm of Garnier et al. (1978; and data not shown). It revealed predominantly α-helical structure. Some of the homologous repeats (α2, α4, α5, α11, α14, α16, α18, α20, and α21) show an α-helical structure without any or with only a slight tendency to breaks. In α1, α3, α6–α9, α12, α13, and α15, on the other hand, several helix-breaking turns and coil structures are predicted. In many repeats they tend to cluster around the positions 58–68 and 80–85.

**Comparison of Various α-Spectrins**

Alignment of the present sequence with the known partial sequences of various α-spectrins is shown in Fig. 5. The published Xenopus oocyte α-spectrin sequence (Giebelhaus et al., 1987) corresponds to the residues 568–1,021, the human fibroblast α-spectrin sequence (McMahon et al., 1987) to the residues 676–1,599, and the rat brain α-spectrin sequence (Leto et al., 1988) to the residues 1,776–2,250. The fragments of the human erythroid α-spectrin sequence (Speicher and Marchesi, 1984) cover scattered parts along the chicken sequence except the COOH terminus.

Comparison between our sequence and the human fibroblast α-spectrin reveals that these sequences are practically identical. It also confirms that the 60-bp insert found in one of the clones of McMahon et al. (1987) represents the predominant transcript and is not a cloning artifact since the same sequence is present here (amino acid residues 1,053–1,073). The observation of McMahon et al. (1987) that there is a 36 amino acid extension in α11 (residues 1,168–1,204 in our sequence) is also corroborated by our sequence.

Xenopus sequence covers only ~17% of the current sequence in the middle part of the molecule. It also shows virtual identity with the chicken brain α-spectrin sequence. Human erythroid α-spectrin has a significantly lower similarity to the chicken brain sequence than the human...
The sequence that is overlined. The authentic clone with a specific primer. was obtained from the genomic clone with a specific primer. Figure 2. Complete nucleotide and amino acid sequences derived from the α-spectrin cDNA CLONES I and II and the genomic CLONE G/I. The amino acid sequence predicted for the long open reading frame is shown with the single letter code. Numbering of the nucleotides begins at the 3' end are underlined. The sequence that is underlined.

**Figure 2.** Complete nucleotide and amino acid sequences derived from the α-spectrin cDNA CLONES I and II and the genomic CLONE G/I. The amino acid sequence predicted for the long open reading frame is shown with the single letter code. Numbering of the nucleotides begins at the 3' end are underlined. The sequence that is underlined.
fibroblast and the frog oocyte spectrins. An unambiguous alignment between its sequenced fragments and the complete brain protein could, however, be made. On that basis a new positioning of the \(\alpha-V\) domain was found. \(\alpha-V\) domain represents the tryptic 41-kD COOH-terminal peptide of erythroid \(\alpha\)-spectrin (T41), and its amino-terminal end had been tentatively placed in the \(\alpha 18\) segment (Speicher and Marchesi, 1984). The present comparison, however, unequivocally places the terminal sequence of this peptide in the \(\alpha 19\) segment as already suggested by Speicher (1986) (the H-RBC peptide placed in \(\alpha 19\); Fig. 5, black arrows). The erythroid spectrin peptide, which was found to be unrelated to the typical repeat by Speicher and Marchesi (1984), finds a match to the boundary of \(\alpha 10\) and \(\alpha 11\). However, a gap has to be introduced in it to allow optimal alignment (\(\alpha 10-\alpha 11\), Fig. 5, asterisks). This gap corresponds to the 60-bp insert of McMahon et al. (1987) (see above).

The partial rat brain \(\alpha\)-spectrin sequence and the present one are again for the most part virtually identical. The rat sequence seems to stop, however, \(\approx 227\) residues short of the end of the chicken brain spectrin. There appears to be a stop codon which corresponds to the nucleotides 6,750–6,752 in our sequence and which causes a termination of the reading frame 681 nucleotides before the stop codon in the chicken sequence. There is a difference in the sequences that brings about this termination: the rat sequence has a T-insert corresponding to the position 6,717 of our sequence. It causes a shift to a frame which contains a TGA stop codon. We think that this may represent a sequencing error, since further downstream in the rat sequence there is an open reading frame coding for protein that would extend the homology to the COOH-terminal part of the chicken brain spectrin (see Discussion).

**Distinct Domains in \(\alpha\)-Spectrin**

Systematic search in protein databases for sequences that are homologous to spectrin indicated four functionally interesting sites in \(\alpha\)-spectrin. Firstly, the COOH terminus of the

### Table I. Quantitation of the Conserved Residues Between the Repeats in Brain \(\alpha\)-Spectrin

| \(\alpha\) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|----------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|
| 1        | X | 19| 23| 18| 18| 50| 17| 26| 22| 5  | 28 | 26| 17| 14| 54| 16| 23| 26| 16| 18| 13| 5  |
| 2        | X | 28| 24| 36| 19| 34| 35| 27| 3  | 42 | 31| 28| 27| 35| 29| 22| 20| 12| 8  |
| 3        | X | 26| 30| 16| 35| 33| 37| 4  | 24| 54| 28| 29| 28| 37| 38| 37| 34| 18| 10| 5  |
| 4        | X | 26| 17| 20| 32| 28| 4  | 14| 26| 56| 24| 25| 24| 32| 24| 19| 18| 16| 3  |
| 5        | X | 20| 28| 37| 6  | 29| 21| 6 | 24| 33| 27| 27| 23| 21| 9 | 7  |
| 6        | X | 18| 23| 21| 5  | 22| 21| 21| 16| 48| 13| 24| 21| 22| 19| 12| 4  |
| 7        | X | 35| 30| 4  | 27| 29| 21| 25| 30| 54| 34| 31| 30| 21| 10| 7  |
| 8        | X | 36| 12| 26| 24| 31| 29| 33| 36| 46| 35| 25| 17| 15| 3  |
| 9        | X | 27| 33| 33| 31| 26| 36| 36| 33| 31| 27| 20| 7 | 3  |
| 10       | X | 5 | 4 | 6 | 5 | 7 | 2 | 3 | 7 | 6 | 5 | 8 | 1  |
| 11       | X | 28| 15| 22| 23| 27| 22| 24| 23| 18| 17| 4  |
| 12       | X | 23| 27| 29| 30| 27| 32| 28| 22| 20| 7  |
| 13       | X | 26| 25| 20| 30| 22| 19| 22| 9 | 4  |
| 14       | X | 21| 28| 24| 26| 29| 17| 9 | 4  |
| 15       | X | 19| 26| 22| 18| 19| 4  |
| 16       | X | 30| 35| 35| 21| 13| 2  |
| 17       | X | 37| 32| 15| 10| 4  |
| 18       | X | 26| 21| 10| 4  |
| 19       | X | 24| 15| 5  |
| 20       | X | 17| 4  |
| 21       | X | 3  |
| 22       | X |    |
chain is clearly related to α-actinin. The latter contains three spectrin repeats as a part of its structure (Wasenius et al., 1987) and shows in its COOH terminus further homology to the carboxy terminus of α-spectrin (Fig. 6). Secondly, within that domain there are two so-called EF-hands (i.e., Ca\textsuperscript{2+}-binding loops) both in α-actinin and α-spectrin (Fig. 7). Before this sequence, there is, thirdly, a segment that could be a calmodulin-binding site. Fourthly, in the middle of the sequence, the distinct unit α10 shows a clear homology to the src-family of protein kinases and to phospholipase C (PLC). 1

Fig. 6 A shows a DIAGON plot that compares the carboxy terminus of α-spectrin (Speicher and Marchesi, 1984). Asterisks, identical residues between neighboring repeats. The four-residue insert (SKHQ) of α15 is excluded from it and placed between the α14 and α15 lines.

In Fig. 7 the putative calcium-binding sequences found in the COOH terminus of spectrin segments α1-α22 are underlined. The optimal alignment of the repeats and the intervening non-homologous regions located by the dot plot in Fig. 3 is shown. The beginning of the first repeat α1 is chosen to fit to the corresponding α1 unit of the human erythroid α-spectrin (Speicher and Marchesi, 1984). Asterisks, identical residues between neighboring repeats. The four-residue insert (SKHQ) of α15 is excluded from it and placed between the α14 and α15 lines.

The alignment of the chicken brain α-spectrin segments α1-α22. The optimal alignment of the repeats and the intervening non-homologous regions located by the dot plot in Fig. 3 is shown. The beginning of the first repeat α1 is chosen to fit to the corresponding α1 unit of the human erythroid α-spectrin (Speicher and Marchesi, 1984). Asterisks, identical residues between neighboring repeats. The four-residue insert (SKHQ) of α15 is excluded from it and placed between the α14 and α15 lines. Vertical lines, the positions of some residues which tend to be conserved in most repeats. These residues are underlined. Nonhomologous regions are overlined. The homology resides in a stretch of ~60 residues which occurs in the amino-terminal half of the src-proteins (see Vyas et al., 1987).

By visual inspection we located in the end of the α21 domain a unique site (residues 2,253-2,371) that contains clusters of basic and hydrophobic residues. By these criteria it might represent a calmodulin-binding site (Kemp et al., 1987). Helical wheel analysis (Fig. 8) reveals its amphipathic nature and net positive charge, which is the consensus drawn for the calmodulin-binding sites in proteins (Erickson-Vitanen and De Grado, 1987).

The α10 domain is homologous to proteins belonging to the family of nonreceptor cytoplasmic tyrosine kinases (src, syn, fgr, lyn, crk, yes, hck, and lsk) as well as to PLC. Fig. 9 shows the optimal alignment of α10 with these sequences. The homology resides in a stretch of ~60 residues which occurs in the amino-terminal half of the src-proteins and in the middle portion of PLC. The degree of similarity ranges from

1. Abbreviations used in this paper: PLC, phospholipase C.
Figure 5. Comparison of the chicken brain α-spectrin (CH-BRAIN; current sequence), Xenopus oocyte α-spectrin (XE-OOCYT; Giebelhaus et al., 1987), human fibroblast α-spectrin (H-FIB; McMahon et al., 1987), rat brain α-spectrin (RAT-BRAIN; Leito et al., 1988), and human red blood cell α-spectrin (H-RBC; Speicher and Marchesi, 1984) sequences. Numbers on the left refer to the repeats of Fig. 4. Asterisks in the boundary of α10–α11 in H-RBC indicate the specific 20-residue insert in the nonerythroid proteins. Black arrows, the H-RBC peptide placed in α19.

Discussion

We present here the nucleotide sequence covering the entire coding frame and the 3' untranslated region plus a part of the 5' flanking sequence of the mRNA for the spectrin α-subunit. The mRNA which was used as a template in the cDNA synthesis was isolated from the embryonic chicken brain. That the derived sequence represents the α-chain of brain spectrin can be deduced from the following: (a) the longest cDNA clone (4.7 kb) isolated from the primary library hybridizes to a probe which in our previous study has been shown to encode a protein that is immunoprecipitated by antibodies specific to α-spectrin, and the sequence of this probe is embedded in the present cDNA sequence; (b) the obtained sequence is more similar to the known α-spectrin than to the β-spectrin sequences (not shown); and (c) it shows a higher degree of homology to the nonerythroid α-spectrin than to the erythroid spectrin sequences.

24 to 32% (identical amino acids) and from 30 to 50% if conservative substitutions are counted. The residues with the consensus sequence ALYDY, KG, and WW in the positions corresponding to 89–92, 104 and 105, and 118 and 119 in src, respectively, are especially well conserved.
The analysis of the current sequence establishes some of the structural principles that have been proposed for the spectrins on the basis of partial and fragmentary sequence information. First of all, the characteristic 106 amino acid repeat forms the basic structural motif of the brain α-spectrin. The remarkably precise conservation of its length in 14 out of 20 homologous units indicates that this preservation is of critical importance for the structure of spectrin. We surmise that the repeats account for the rod-like shape of the molecule and that the evolutionary constraint to preserve the 106 amino acid length is imposed by the formation of multiple contacts.
Figure 9. Homology of the α10 domain to the src-proteins and src-like proteins and to PLC. On the top, the chicken src tyrosine kinase and α-spectrin are schematically aligned to illustrate the location of this homologous region (3) in their linear maps. Modulatory and kinase refer to the two domains in the src-proteins. The homologous sequences are shown on the bottom. The numbers on the left refer to the first residues taken to the alignment. Asterisks and vertical lines indicate the identical and conservative substitutions, respectively, between α10 and each of the sequences. The two numbers on the right are taken from these pairwise comparisons and, again, refer to the number of identical (asterisks) and total conserved (asterisks and vertical lines) residues. The sequences are taken from the following references: c-src, Takeya and Hanafusa, 1982; fyn, Kawakami et al., 1986 and Semba et al., 1986; v-src, Takeya and Hanafusa, 1982 and Taylor and Hanafusa, 1983; fgr, Katamine et al., 1988; lyn, Yamanashi et al., 1987; c-yes, Sukegawa et al., 1987; hck, Quintrell et al., 1987; Isk, Marth et al., 1985; tkl, Strebhardt et al., 1985; v-crk, Mayer et al., 1988; PLC, Stahl et al., 1988.
with \( \beta \)-spectrin, which possesses the basically similar structural principle (Speicher and Marchesi, 1984).

In the chicken brain \( \alpha \)-spectrin we can discern 18 repeats (\( \alpha_1\)–\( \alpha_9 \), \( \alpha_{11}\)–\( \alpha_{19} \)) with a relatively high degree of homology, two repeats (\( \alpha_{20}, \alpha_{21} \)) with a lower degree of homology, and the \( \alpha_{10} \) and \( \alpha_{22} \) segments as well as the 36 residue extension of the \( \alpha_{11} \) that are unrelated to the homologous repeats. This divides the chain into 22 segments (Fig. 10).

Comparison of the present sequence with the published spectrin sequences corroborated the early observation (see McMahon et al., 1987 for detailed analysis) that the nonerythroid \( \alpha \)-spectrins from different species, ranging in this study from Xenopus to man, show a high degree of mutual homology while the mammalian erythroid \( \alpha \)-spectrin is a more distant protein. This indicates a rapid divergent evolution of the latter.

In accordance with McMahon et al. (1987) we found that the \( \alpha_{10} \) and \( \alpha_{11} \) units contain sequences unrelated to the homologous repeats. Interestingly, the homology of these segments between chicken and man is not different from that of the other domains of spectrin. This suggests that the distinct characteristics of these domains were established before the divergence of the human and avian species. Comparison of these with the corresponding regions of the erythroid spectrin is hampered by the paucity of sequence data. Interestingly, however, it seems that the amino acids encoded by the 60 nucleotide insert in the nonerythroid spectrins are lacking in the erythroid spectrin (McMahon et al., 1987) indicating a specific function for the \( \alpha_{10} \)–\( \alpha_{11} \) domain in nonerythroid cells.

The mol mass calculated for our sequence is 285,369. This is \( \sim10\% \) higher than the highest estimates based on SDS-PAGE (Bennett et al., 1982). The 22 segments of the brain \( \alpha \)-spectrin are two more than predicted for the erythroid \( \alpha \)-spectrin by Speicher and Marchesi (1984). This discordance may be due to a mispositioning of the NH\(_2\) terminus of tryptic fragment T4I, belonging to the domain \( \alpha_V \), in \( \alpha_{18} \) instead of \( \alpha_{19} \) (see above; Speicher, 1986). If this is taken into account in the modeling of the molecule, the number of the repeats in the human erythroid spectrin amounts to 21.

We suggest that there is another (nonhomologous) segment in the COOH terminus of erythroid \( \alpha \)-spectrin which corresponds to our \( \alpha_{22} \). This is based on the following calculation: positioning of the amino terminus of the T4I in the beginning of the \( \alpha_{19} \) would give erythroid \( \alpha \)-spectrin a molecular mass of \( \sim260 \) kD (the calculated molecular mass of the \( \alpha_{10} \)–\( \alpha_{11} \) domain structure.

![Figure 10. A schematic map for the \( \alpha \)-spectrin domain structure.](image)
the NH$_2$ terminus of the β-chain with the COOH terminus of the α-chain exerting control on the interaction. That spectrin can, indeed, bind actin in a calcium-dependent manner, is demonstrated by the recent finding of Fishkind et al. (1987) showing modulation of the spectrin-actin interaction by calcium in sea urchin egg.

Spectrin is also involved in other calcium-regulated events such as the complex formation with calpain (Gerke and Weber, 1984) and the degradation by a Ca$^{2+}$-regulated protease (Siman et al., 1984). Furthermore, brain spectrin can also modulate the Mg$^{2+}$-ATPase activity of the smooth muscle actomyosin in a calcium-dependent manner (Wagner, 1984; Wang et al., 1987). This calcium sensitivity may at least partially be conferred by Ca$^{2+}$ binding to the EF-hand structures in α22.

Nonerythroid spectrins are marked by their capacity to bind calmodulin to their α-subunit (Glenney et al., 1982a, b; Kakiuchi et al., 1982; Palfrey et al., 1982). The exact location of the binding site is, however, not resolved. In the present sequence there is a domain in α21 that fulfills the structural criteria proposed for calmodulin-binding sites. Such a positioning is supported by Carlin et al. (1983) who found that the major proteolytic breakdown product of fodrin (spectrin), which represents the COOH-terminal part of the chain is able to bind calmodulin. Tsuchita et al. (1983) have, however, suggested that the calmodulin-binding site is close to the amino terminus of the α-chain. Subcloning and expression of the cDNA containing the putative calmodulin-binding site will enable us to test our proposal.

An intriguing finding from the homology search was the similarity between a defined region in α10, in PLC, and in the amino-terminal half of the α-subunit. The members of the “src-subfamily” are oncogenes and all, except for crk, have kinase activity (Hanks et al., 1988; Mayer et al., 1988), a feature that has not been associated with spectrin. On the other hand, many of these proteins (Hunter and Cooper, 1985) are closely associated with the cytoplasmic side of the plasma membrane. In this regard these proteins are similar to spectrin.

The function of the α10 domain in spectrin is currently unknown. Similarly, the specific functions of the homologous domains in the src-proteins and PLC have not been elucidated. It is clear, however, from the studies with deletion mutants, that the NH$_2$-terminal portion of the src-proteins is not needed for the tyrosine kinase activity. On the other hand, the NH$_2$-terminal half has a modifying effect on the intrinsic kinase activity that resides in the COOH-terminal half (Jove and Hanafusa, 1987). The NH$_2$-terminal half has been suggested to recognize or bind the substrates which are then phosphorylated by the COOH-terminal catalytic domain. Hence, the term modulatory (Calothy et al., 1987) or recognition (Parsons et al., 1984) domain has been coined to the NH$_2$ terminus of the src.

Using this analogy, we may suggest that the α10 domain in spectrin could serve as a recognition site for some substrates of src tyrosine kinases. One candidate could be calpain I, also known as p36, which interacts with α-spectrin (Lehto et al., 1983; Gerke and Weber, 1984) and which is also a major substrate of src tyrosine kinases (Hunter and Cooper, 1985). Alternatively, the homologous regions in spectrin, PLC, and src-proteins could anchor these proteins to some common cytoskeletal component. This would explain the reduced association of src-protein with cytoskeleton when they are deleted for the NH$_2$-terminal modulatory domain (Hamaguchi and Hanafusa, 1987).

**Evolution of Spectrin**

It is evident that spectrin has evolved from an ancestral gene coding for the basic 106-residue repeat by several contiguous duplications (Speicher and Marchesi, 1984). In our view, the ancestral repeat unit has first undergone three duplication steps to reach an 8-repeat stage. Possibly at this stage there has also been insertion of one more repeat to make a 9-repeat structure which has then undergone another, final duplication. Concurrently, the gene has also acquired the non-homologous domains possibly by exon shuffling. The elucidation at what stage during evolution these divergent regions have been assimilated by spectrin—whether spectrin has acquired new functions during its recent evolutionary history—has to await primary structures of spectrins from lower organisms. The spectrin repeat has also been found in two other proteins, α-actinin (Wasenius et al., 1987) and dystrophin (Koenig et al., 1988). Hence, these proteins may have evolved from a common ancestor and possess a similar architectural design.

The present cDNA sequence of α-spectrin establishes that the same basic structural principle is found in various types of spectrin. It also clearly indicates regions which may carry some important functions of spectrin. Further studies to test the functional properties of these domains are now greatly facilitated by the availability of cDNA clones; fragments of the structure can now be expressed separately, and the produced polypeptides studied experimentally for the postulated functions.

The authors thank Ms. Mirja Leppäkoski for making the synthetic oligonucleotides; Ms. Hilkka Penttinen, Ms. Outi Rauanheimo, and Ms. Marjaleena Rissangan for typing the manuscript; and Mr. Pekka Mäkinen for helping with computers.

This study was supported by the Academy of Finland, the Sigrid Juselius Foundation, and the Finnish Cancer Institute.

Received for publication 28 March 1988, and in revised form 29 August 1988.

**Note added in proof**: Recently Harris et al. (Harris, A. S., D. E. Croall and J. S. Morrow. 1988. J. Biol. Chem. 263:15754–15761) have shown that one calmodulin-binding site in human fodrin resides in the terminal portion of the 11th repeat.

**References**

Baron, M. D., M. D. Davison, P. Jones, and D. R. Critchley. 1987. The sequence of chick α-actinin reveals homologies to spectrin and calmodulin. J. Biol. Chem. 262:17623–17629.

Bennett, V., J. Davies, and W. E. Fowler. 1982. Brain spectrin: a membrane-associated protein related in structure and function to erythrocyte spectrin. Nature (Lond.). 299:126–131.

Biggin, M. D., T. J. Gibson, and G. F. Hong. 1983. Buffer gradient gels and 35S-labeling as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA. 80:3963–3965.

Birkenmeier, C. S., D. M. Bodine, E. A. Repasky, D. M. Helfman, S. H. Hughes, and J. E. Barker. 1985. Remarkable homology among internal repeats of erythroid and nonerythroid spectrin. Proc. Natl. Acad. Sci. USA. 82:5671–5675.

Burns, N. R., V. Ohtman, and W. B. Gratzer. 1983. Properties of brain spectrin (fodrin). FEBS (Fed. Eur. Biochem. Soc.) Lett. 153:165–168.

Burridge, K., T. Kelly, and P. Mangeat. 1982. Nonerythocyte spectrins: actin-membrane attachment proteins occurring in many cell types. J. Cell Biol. 95:478–486.

Calothy, G., D. Laugier, R. F. Cross, R. Jove, T. Hanafusa, and H. Hanafusa. 1987. The membrane-binding domain and myristylation of p60s is not es-
senital for stimulation of cell proliferation. *J. Virol.* 61:1678–1681.

Carlin, R. K., D. C. Bartelt, and P. Sievek. 1983. Identification of fodrin as a major calmodulin-binding protein in postcytic dense preparations. *J. Cell Biol.* 96:443–448.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonuclease from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

Erickson-Vitanen, S., and W. F. De Grado. 1987. Recognition and characterization of calmodulin-binding sequences in peptides and proteins. *Methods Enzymol.* 139:455–475.

Fiskhjelm, D. J., E. M. Bondar, and D. A. Beeg. 1987. Isolation and characterization of a sea urchin egg spectrin: calmodulin modulation of the spectrin-cytoskeletal link. *Cell Motil. Cytoskeleton.* 7:304–314.

Garnier, J. I., D. J. Osagbuchen, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* 120:97–120.

Gebel, V. 1984. Identity of p36K phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders: calcium-dependent binding to non-erythroid spectrin and F-actin. *EMBO (Eur. Mol. Biol. Organ.)* J. 3:227–231.

Gebel, D. H., B. D. Zelus, S. K. Henschman, and R. T. Moon. 1987. Changes in the expression of a-fodrin during embryonic development of Xenopus laevis. *J. Cell Biol.* 105:843–853.

Glenney, J. R., Jr., and P. Glenney. 1984a. Fodrin is the general spectrin-like main proteins. *J. Cell Biol.* 107:167–275.

Glenney, J. R., Jr., and P. Glenney. 1984b. Comparison of spectrin isolated from erythroid and non-erythroid sources. *Eur. J. Biochem.* 144:529–539.

Glenney, J. R., Jr., P. Glenney, M. Osborn, and K. Weber. 1982a. An F-actin crogenic potential of a novel human p55 c-fgr.

Glenney, J. R., Jr., P. Glenney, and K. Weber. 1982b. Erythroid spectrin: calmodulin modulation of the spectrin-cytoskeletal link. *Cell Motil. Cytoskeleton.* 7:304–314.

Glenney, J. R., Jr., and P. Glenney. 1984b. Comparison of spectrin isolated from erythroid and non-erythroid sources. *Eur. J. Biochem.* 144:529–539.

Glenney, J. R., Jr., P. Glenney, M. Osborn, and K. Weber. 1982a. An F-actin crogenic potential of a novel human p55 c-fgr.

Glenney, J. R., Jr., P. Glenney, and K. Weber. 1982b. Erythroid spectrin: calmodulin modulation of the spectrin-cytoskeletal link. *Cell Motil. Cytoskeleton.* 7:304–314.

Glenney, J. R., Jr., and P. Glenney. 1984b. Comparison of spectrin isolated from erythroid and non-erythroid sources. *Eur. J. Biochem.* 144:529–539.

Glenney, J. R., Jr., P. Glenney, M. Osborn, and K. Weber. 1982a. An F-actin crogenic potential of a novel human p55 c-fgr.

Glenney, J. R., Jr., and P. Glenney. 1984b. Comparison of spectrin isolated from erythroid and non-erythroid sources. *Eur. J. Biochem.* 144:529–539.

Glenney, J. R., Jr., and P. Glenney. 1984b. Comparison of spectrin isolated from erythroid and non-erythroid sources. *Eur. J. Biochem.* 144:529–539.

Glenney, J. R., Jr., P. Glenney, M. Osborn, and K. Weber. 1982a. An F-actin crogenic potential of a novel human p55 c-fgr.

Glenney, J. R., Jr., P. Glenney, M. Osborn, and K. Weber. 1982a. An F-actin crogenic potential of a novel human p55 c-fgr.
Sequence similarity of phospholipase C with the non-catalytic region of src. Nature (Lond.). 332:269–272.
Strebhardt, K., J. I. Mullins, C. Bruck, and H. Rübsamen-Waigmann. 1985. Additional member of the protein-tyrosine kinase family: the src- and icl-related proto-oncogene c-ıklı. Proc. Natl. Acad. Sci. USA. 84:8778–8782.
Sukegawa, J., K. Semb, Y. Yamanashi, M. Nishizawa, N. Miyajima, T. Yamamoto, and K. Toyoshima. 1987. Characterization of cDNA clones for the human c-yes gene. Mol. Cell. Biol. 7:41–47.
Takeya, T., and H. Hanafusa. 1982. DNA sequence of the viral and cellular src gene of chicken. II. Comparison of the src genes of two strains of avian sarcoma virus and of the cellular homolog. J. Virol. 44:12–18.
Taylor, T., and H. Hanafusa. 1983. Structure and sequence of the cellular gene homologous to the RSV src gene and the mechanism for generating the transforming virus. Cell. 32:881–890.
Tsukita, S., S. Tsukita, H. Ishikawa, M. Karokawa, K. Morimoto, K. Sobue, and S. Kakiuchi. 1983. Binding sites of calmodulin and actin on the brain spectrin, calspectin. J. Cell Biol. 97:574–578.
Tufty, R. M., and R. H. Kretsinger. 1975. Troponin and parvalbumin calcium regions predicted in myosin light chain and T4 lysozyme. Science (Wash. DC). 187:167–169.
Virtanen, I., L. E. Thornell, I. Damjanov, M. Hormia, and V. P. Lehto. 1986. Distribution of erythroid alpha-spectrin analogs in mammalian cells and tissues. Membrane skeleton and cytoskeletal–membrane association. J. Cell Biochem. 30:339–356.
Vyas, N. K., M. N. Vyas, and F. A. Quiocho. 1987. A novel calcium-binding site in the galactose-binding protein of bacterial transport and chemotaxis. Nature (Lond.). 327:635–638.
Wagner, P. D. 1984. Calcium-sensitive modulation of the actomyosin ATPase by fodrin. J. Biol. Chem. 259:6306–6310.
Wang, C., P. K. Ngai, M. P. Walsh, and J. H. Wang. 1987. Ca2+- and calmodulin-dependent stimulation of smooth muscle actomyosin Mg2+-ATPase by fodrin. Biochemistry. 26:1110–1117.
Wasenius, V.-M., O. Näränen, V.-P. Lehto, and M. Saraste. 1987. α-Actinin and spectrin have common structural domains. FEBS (Fed. Eur. Biochem. Soc.) Lett. 221:73–76.
Wasenius, V.-M., M. Saraste, J. Knowles, I. Virtanen, and V.-P. Lehto. 1985. Sequencing of the chicken non-erythroid spectrin cDNA reveals an internal repetitive structure homologous to the human erythrocyte spectrin. EMBO (Eur. Mol. Biol. Organ.) J. 4:1425–1430.
Yamanashi, Y., S. I. Fukushige, K. Semb, J. Sukegawa, N. Miyajima, K.-I. Matsubara, T. Yamamoto, and K. Toyoshima. 1987. The yes-related cellular gene lyn encodes a possible tyrosine kinase similar to p56lck. Mol. Cell. Biol. 7:237–243.