Sequencing of the chicken non-erythroid spectrin cDNA reveals an internal repetitive structure homologous to the human erythrocyte spectrin

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Immunological screening of a chicken gizzard cDNA expression library was used to isolate two clones encoding a part of the non-erythroid spectrin-like protein. Clones were identified by immunoblotting of the polypeptides synthesized in *Escherichia coli* cells transformed with cDNA cloned in the pUC8 plasmid vector using polyclonal rabbit antibodies raised against bovine non-erythroid spectrin. The sequence of an ~1.5-kb cDNA insert of one clone was determined. Analysis of the predicted amino acid sequence reveals that, despite differences in immunological cross-reactivity and peptide maps, the chicken non-erythroid and the human erythrocyte spectrins are highly homologous proteins. Like the human erythrocyte spectrin, the chicken smooth muscle spectrin appears also to be constructed from repeated, homologous structures of 106 amino acid residues. This is probably a universal structure motif of spectrins.
Key words: spectrin/cDNa/amino acid sequence/non-erythroid

Introduction

Spectrin is the major constituent of the cytoskeletal network underlying the erythrocyte plasma membrane and, until recently, was considered to be specific for red blood cells (Marchesi, 1979; Branton *et al.*, 1981). It is composed of two non-identical polypeptides termed α- and β-spectrin. The heterodimer self-associates to an $(\alpha,\beta)_2$-tetramer through linkage with actin and a protein called band 4.1 (Branton *et al.*, 1981). This complex is anchored to the cytoplasmic face of the plasma membrane *via* another protein, ankyrin, which binds to β-spectrin and mediates the binding of the whole complex to a transmembrane protein band 3 (Bennett and Stenbuck, 1979; Branton *et al.*, 1981). The interactions of erythrocyte spectrin with other proteins through specific binding domains (Morrow *et al.*, 1980) lead to the formation of an extensive subplasmalemmal meshwork which is thought to be responsible for the maintenance of the biconcave shape of human erythrocytes, for the regulation of plasma membrane components and for the maintenance of the lipid asymmetry of the plasma membrane (for a review, see Goodman and Shiffer, 1983; Cohen, 1983).

Counterparts of the erythrocyte spectrin, by the criteria of immunological cross-reactivity and some physical and functional properties, were recently found in non-erythroid cells. Such spectrin-like proteins (also termed immunoanalogues of spectrin) have been demonstrated in a variety of avian and mammalian non-erythroid cells and tissues (Goodman *et al.*, 1981; Levine and Willard, 1981; Bennett *et al.*, 1982; Glenney *et al.*, 1982a;

Repasky *et al.*, 1982; Glenney and Glenney, 1983b; for a review, see Lazarides and Nelson, 1982; Kakiuchi and Sobue, 1983; Baines, 1983). They frequently show immunological cross-reactivity to antibodies raised against chicken erythrocyte α-spectrin, but usually do not cross-react with antibodies against mammalian erythrocyte α-spectrin (Lazarides and Nelson, 1982). It is now well established that spectrin-like proteins from different sources differ in their subunit composition (Glenney and Glenney, 1983a, 1983b; Glenney *et al.*, 1983). Mammalian erythroid spectrin in particular is highly divergent from the other members of the family to judge from its immunological cross-reactivity and peptide map (Glenney and Glenney, 1984). This probably reflects cell-type specific interactions and functions carried out by the network of spectrin-like proteins in different types of cells (Nelson *et al.*, 1983; Nelson and Lazarides, 1983; Lazarides *et al.*, 1984).

Like erythrocyte spectrin, the spectrin-like proteins from other sources have high mol. wts. (200−260 kd) and are capable of forming dimers which can further associate to tetramers (Glenney *et al.*, 1982a, 1982b; Burridge *et al*; 1982). Morphologically, spectrin-like proteins appear to be related but not identical molecules showing a flexible rod-like structure (Glenney *et al.*, 1982a, 1982b). They can also bind actin (Glenney *et al.*, 1982a, 1982b; Carlin *et al.*, 1983) but seem to differ in their calmodulin-binding activity (Glenney and Glenney, 1984). In immunofluorescence and immunoelectron microscopy, non-erythroid spectrins, like spectrins in red cells, are found to be located at the cell periphery in close apposition to plasma membranes (Levine and Willard, 1981; Lehto and Virtanen, 1983; Virtanen *et al.*, 1984). An exception is the spectrin-like protein TW 260/240 which is located in the terminal web of the chicken intestinal epithelium (Glenney *et al.*, 1982a). Thus, although they display significant differences, these proteins seem to be structurally and functionally related, and have, therefore, often been considered an evolutionarily related family of proteins (Glenney and Glenney, 1983a, 1984; Goodman *et al.*, 1983).

Establishment of the structural and evolutionary relationships in the spectrin family has been hampered by the lack of sequence data. Recently, however, Speicher *et al.* (1983) and Speicher and Marchesi (1984) have published a partial amino acid sequence of the human erythrocyte spectrin. It reveals an unusual structure of multiple repeats and proposes a model for the human erythrocyte spectrin in which 20 repetitive units are found in the α-chain and 18 in the β-chain. Each unit has 106 amino acid residues. Secondary structure prediction (Chou and Fasman, 1978) proposes that there are three long α-helical segments in each unit. This model depicts human erythrocyte spectrin as a string of pearls, the triple helical structures being connected by flexible strings (Speicher and Marchesi, 1984).

Here we report a part of the amino acid sequence of the chicken non-erythroid α-spectrin. This has been predicted from the nucleotide sequences of a clone isolated from a cDNA expression library of chicken smooth muscle mRNA. This protein sequence and its comparison with the partial amino acid sequence of human

erythrocyte spectrin shows that the chicken non-erythroid spectrin and the human erythrocyte spectrin are clearly homologous. The 106-residue repetitive unit is also found in the chicken non-erythroid spectrin and we suggest it is a universal structure motif of all spectrin-like proteins.

Results

Identification of the non-erythroid spectrin cDNA clone

The construction of a cDNA library from 11-day embryonic

chicken gizzard and stomach mRNA in the expression vector pUC8 has been described previously (Helfman *et al.*, 1983). The library was screened for spectrin-like proteins by incubating replica filters with anti-p230 antibodies essentially as described by Helfman *et al.* (1983). The anti-p230 antibodies identified two clones 18-3a and 18-3b. In both, the inserted cDNA is about the same size: ~ 1.5 kb. One of these, 18-3a, was analysed further. Polypeptides in the mol. wt. region of 55 000 daltons were found in immunoblotting with anti-p230 antibodies of the proteins syn-
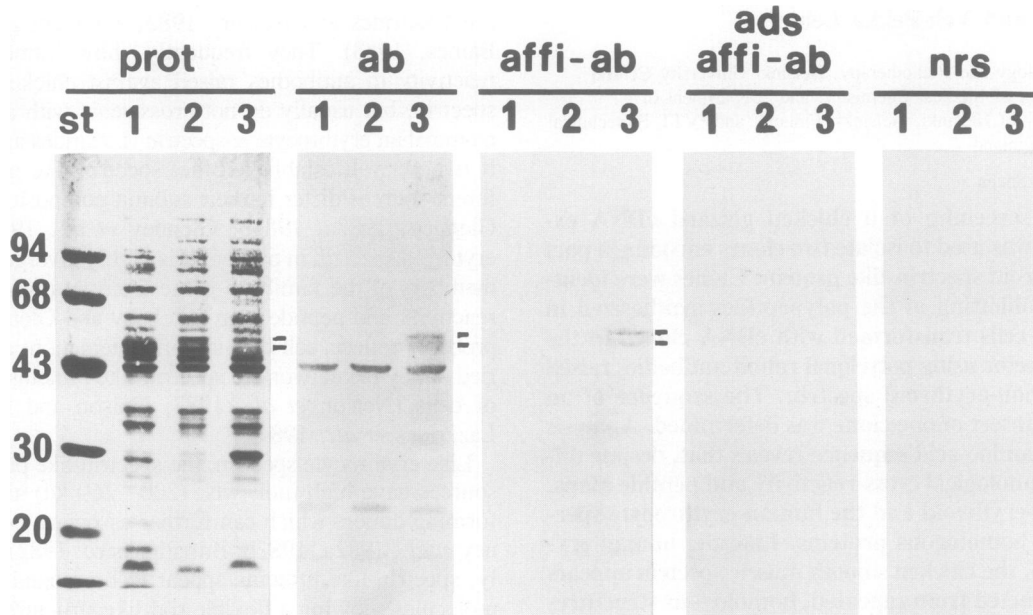

Fig. 1. Immunoblots of lysates of DH-1 cells (**slots 1**), DH-1 cells transformed with pUC8 plasmid (**slots 2**) and DH-1 cells transformed with pUC8 plasmid with a chicken smooth muscle cDNA insert encoding a polypeptide reactive with anti-p230 antibodies (anti-p230) (**slots 3**). *Prot*, polypeptides transferred to nitrocellulose paper and stained with amido black; *ab*, immunoblotting with anti-p230 antiserum; *affi-ab*, immunoblotting with anti-p230 antibodies affinity-purified with nitrocellulose-bound chicken erythroid α-spectrin (for details, see Lehto and Virtanen, 1983); *ads affi-ab*, immunoblotting with affinity-purified anti-p230 antibodies pre-adsorbed with purified chicken erythrocyte spectrin; *nrs*, immunoblotting with rabbit non-immune serum; *st*, mol. wt. standard (indicated in kd). The position of the anti-p230-reactive polypeptide is indicated with small bars.
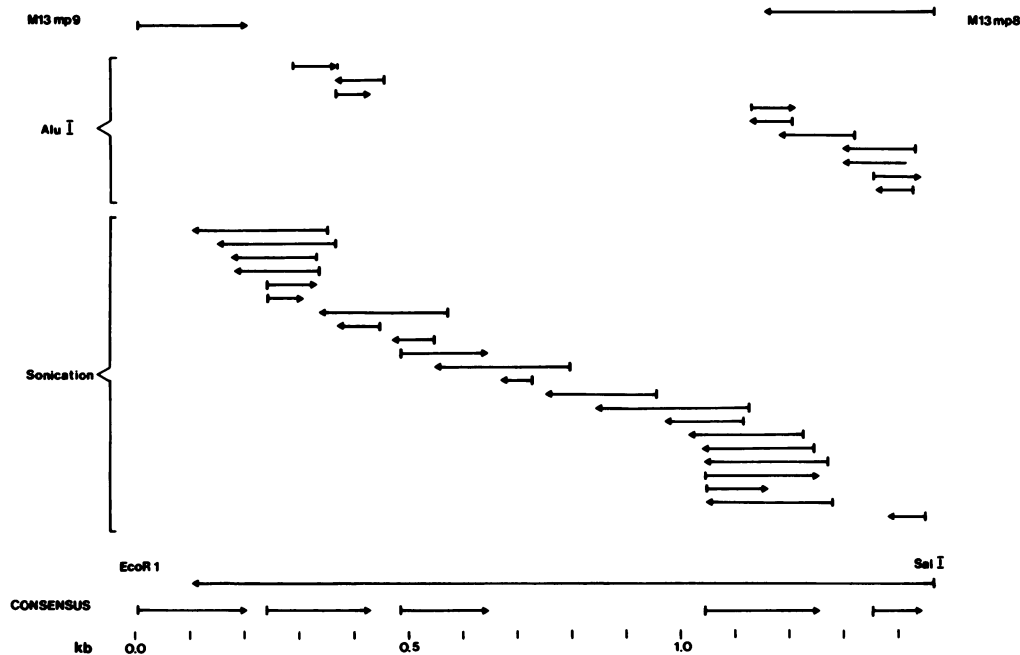

Fig. 2. Sequencing strategy. The arrows indicated determined sequences. 'Sonication' refers to fragments cloned from the sonicated DNA and '*Alu*I' to fragments from the *Alu*I-digested DNA. 'M13mp8' and 'M13mp9' refer to the sequences determined from the ends of the entire 1.5-kb fragment cloned into these two vectors. 'Consensus' summarises the coverage of the two strands.

thesised in the *E. coli* cells containing the recombinant plasmid 18-3a (Figure 1). The upper band is most likely the cDNA-encoded protein and the lower one a degradation product. No staining of the blots was seen with anti-p230 antibodies pre-adsorbed with the purified chicken erythrocyte α-spectrin, or with normal (non-immune) rabbit serum.

Nucleotide sequence of the clone 18-3a

Shotgun cloning of the DNA fragments produced by sonication or digestion with *Alu*I covered almost the entire 1.5-kb sequence. The *Eco*RI and *Sal*I ends were sequenced after cloning the whole fragment in M13mp8 and mp9 in both orientations. This sequencing strategy is summarized in Figure 2. The complementary strand was recovered by 88% in the consensus sequence, while only 57% of the coding strand was determined.

The complete sequence of 18-3a cDNA is shown in Figure

3. It includes the 5' end *Eco*RI and 3' *Sal*I sites which may originate from the linkers in cDNA cloning (Helfman *et al.*, 1983). The predicted amino acid sequence of 478 residues is shown between the *Eco*RI and *Sal*I sites.

Analysis of the predicted amino acid sequence

A systematic comparison using the computer program DIAGON (Figure 4) of the published partial sequence of the human erythrocyte α-spectrin (Speicher and Marchesi, 1984) to the 478 residues of the chicken non-erythrocyte spectrin shows that (i) these two proteins are homologous and (ii) both are made of repetitive units of 106 amino acid residues (Figure 4). In the sequence reported here there are three complete and two incomplete repeats. The last one of them seems to differ from the others in that there is apparently an insert between positions 28 and 29 and 60 and 61 (Figure 5a). Because the structures are repetitive
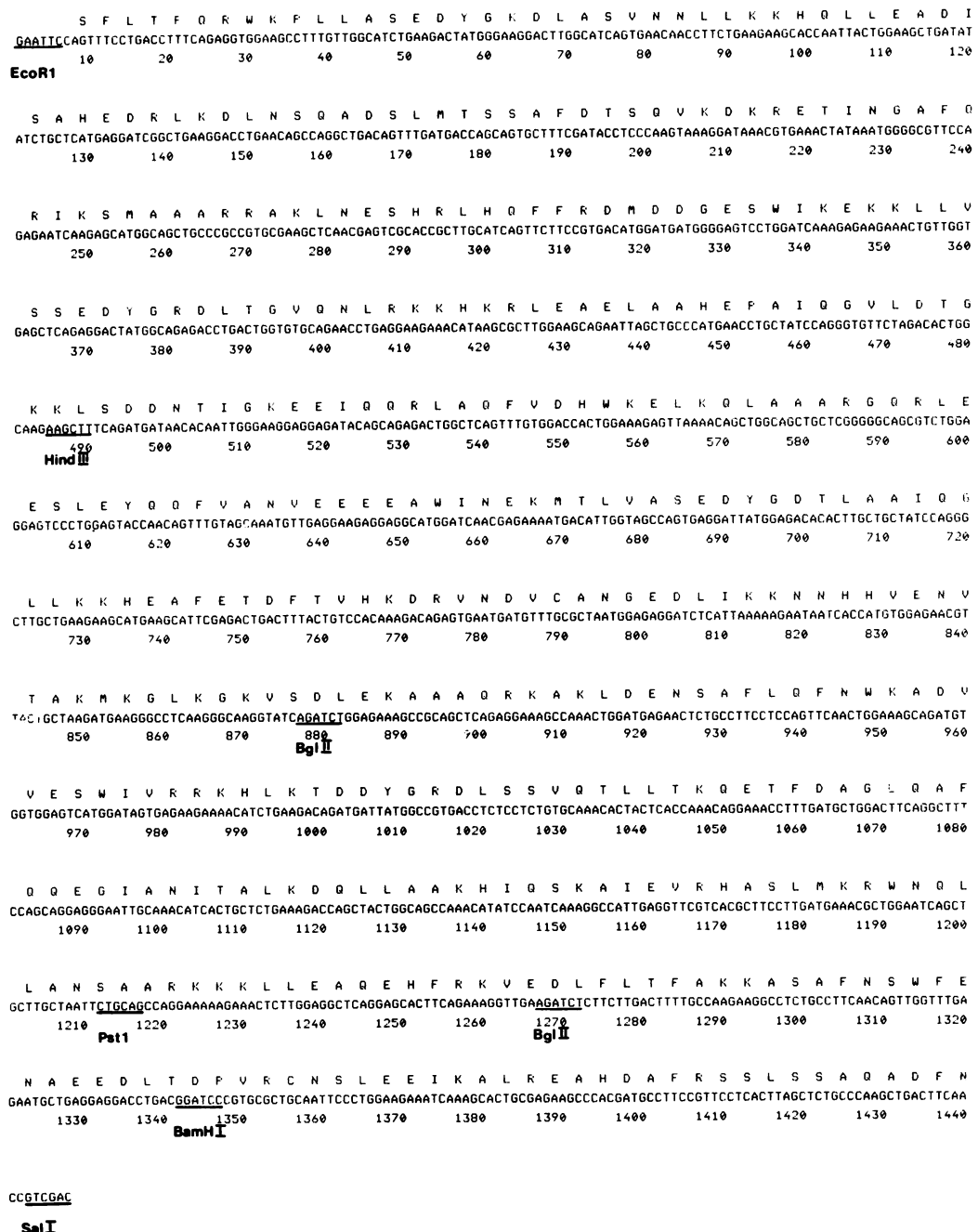

Fig. 3. Nucleotide sequence of the cDNA clone 18-3a. The *Eco*RI and *Sal*I sites at the 5' and 3' ends as well as several other indicated restriction sites are underlined.
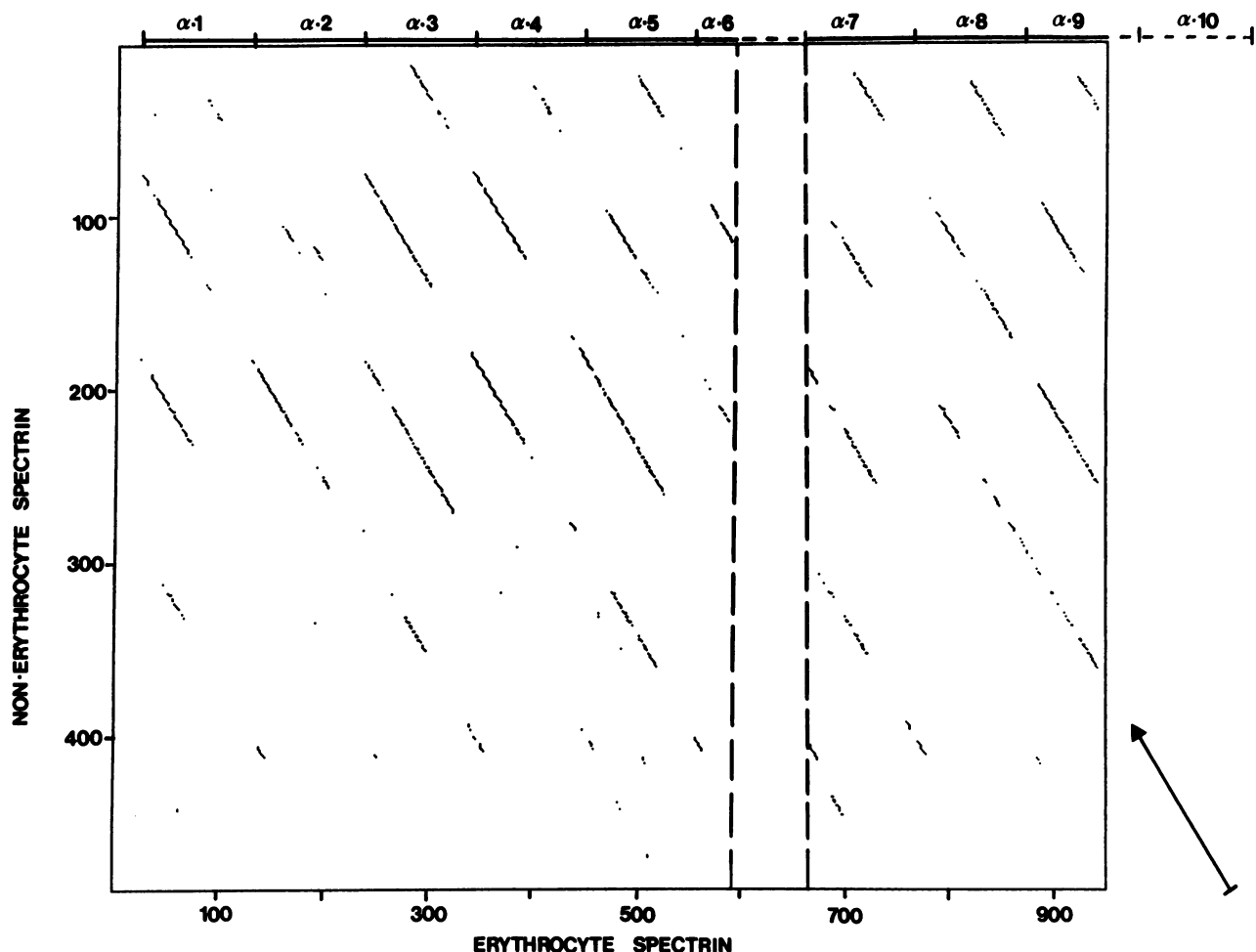
Fig. 4. Sequence homology was searched using a computer program DIAGON where scoring is based on a mutation data matrix (see Staden, 1982). The span used in calculation was 21 and the dots shown have a score 242 corresponding to the double matching probability (McLachlan, 1971) of 5×10^{-5}. The arrow shows the proposed alignment of the two proteins (see text). Comparison of the erythrocyte and non-erythrocyte α-spectrin. The horizontal axis is the partial N-terminal amino acid sequence of the human red cell α-spectrin. The labelling of the repeats α-1 to α-10 is taken from Speicher and Marchesi (1984). There is a cap in the sequence between residues 592 and 654 which is shown by the dashed lines. The vertical axis is the predicted amino acid sequence of the chicken non-erythroid spectrin taken from Figure 3.
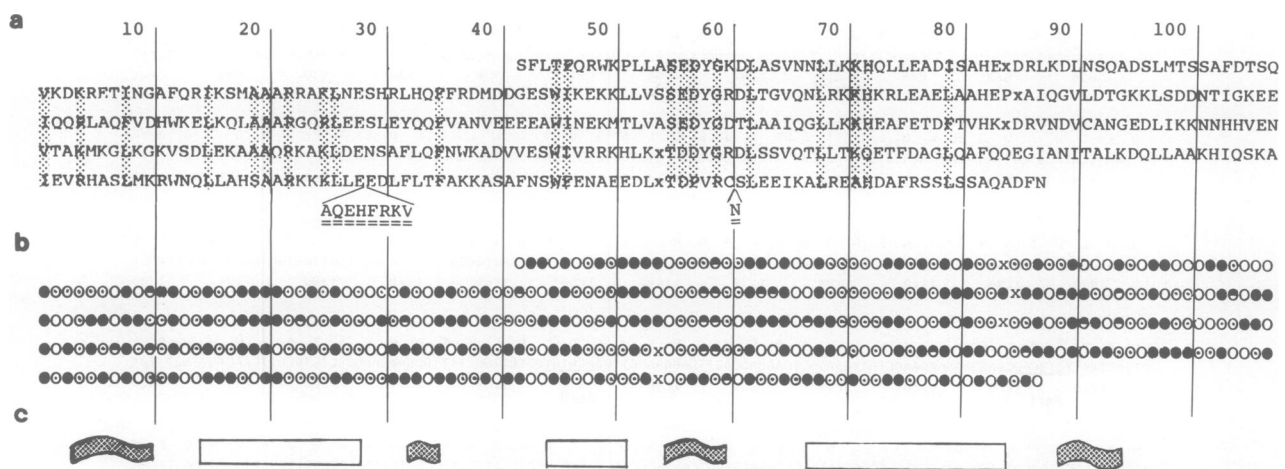

Fig. 5. The repetitive units in the non-erythroid spectrin (a). The sequence taken from Figure 3 is arranged similarily to that in Speicher and Marchesi (1984). Shaded rows indicate positions where a similar residue tends to occur in the repeats of the non-erythrocyte α-spectrin as well as in the erythrocyte α-spectrin. Eight underlined residues between positions 28 and 29 and one between 60 and 61 are inserts belonging to the last repeat. (x) refers to a deletion. A plot indicating the positions occupied by hydrophobic and charged residues (b). Hydrophobic residues: ● I, V, W, L, M, A, P and ◐ Y, C, G. Charged residues: ⊙ E, D, K, R, H. Secondary structure prediction of the repeats (c). Empty boxes and shaded ribbons below the sequence mark the probable α-helical and β-turn structures commonly predicted for all the repeats. Secondary structure was predicted using the Chou-Fashman procedure and a span of four amino acid residues in the calculation.

and only ~25% of the proteins has been sequenced, it is not yet possible to find an unambiguous alignment of the human erythrocyte and chicken non-erythroid spectrin. There is, however, one particularly interesting alignment. It has been suggested that the α-10 unit in the middle of the α-chain differs from the others in the human red cell spectrin (Speicher and Marchesi, 1984). We assume also that the last repeat in our sequence is different from the others and may thus correspond to the α-10 unit of the erythrocyte spectrin. This alignment is indicated by the arrow in Figure 4. In this alignment there is a 25% identity between the existing common sequence of the human erythrocyte and the chicken non-erythroid spectrins (not shown).

The alignment presented in Figure 5a shows that in several positions an invariant or conservatively substituted amino acid residue occurs. These positions are predominantly occupied by the same or a similar residue in the alignment of repeats of the human erythrocyte spectrin (Speicher and Marchesi, 1984). Thus tryptophan is found in position 45, histidine in position 72, leucine in position 15 and 26, phenylalanine in position 35, isoleucine in position 46, arginine in position 22 and lysine in position 71. This homology is further sustained by the closely similar pattern of hydrophobic and charged residues in the two sequences (Figure 5b). As in the case of human erythrocyte spectrin, here also the amino-terminal ends of the repeats appear to be more strongly conserved than the carboxy-terminal ends.

Secondary structure prediction (Chou and Fasman, 1978) suggests that each repeat of the predicted chicken non-erythroid spectrin contains two long α-helical structures and a third shorter α-helix. Location of these together with the strongly predicted β-turns, common to all repeats, is shown in Figure 5c. This prediction resembles closely that derived for the repeats in the human red cell spectrin, which is known to have a high α-helical content (see Speicher and Marchesi, 1984).

Discussion

Immunological screening of the 100 000 recombinant colonies with the anti-spectrin antibodies detected two positive clones. The low yield is consistent with the observation that spectrin is not an abundant protein in smooth muscle cell (Repasky et al., 1982). Additional factors which may interfere either with the expression or with the detection of the spectrin-related products are the toxicity of some translation products to the host cells, insertion of the cDNA in an incorrect reading frame or expression of the mRNA/cDNA fragment that does not possess epitopes recognizable with the anti-p230 antibodies used in this study.

Numerous immunological and electrophoretic studies have established that the mammalian erythrocyte spectrins diverge most from the family of spectrin-like proteins. They are clearly distinct from the mammalian and avian non-erythroid spectrins and also from the avian erythrocyte spectrins (Glenney and Glenny, 1983a, 1983b, 1984). Comparison of the proteolytic peptide maps of these two types of proteins has failed to reveal any common structural principle in their domain organization. On the other hand, avian erythrocyte spectrins and avian and mammalian non-erythroid spectrins show a high degree of structural homology and immunological cross-reactivity (Bennett et al., 1982; Lazarides and Nelson, 1982; Glenney and Glenney, 1983a, 1984; Nelson et al., 1983).

The partial amino acid sequence presented in this study is the first insight into the primary structure of the non-erythroid spectrin-like proteins and offers a possibility of comparing the primary structure of human erythrocyte and chicken non-erythroid spectrins. Furthermore, it enables evaluation of the

alleged evolutionary relatedness of these two types of proteins which thus far has been based on structural, functional and immunological similarities.

The results show that the predicted amino acid sequence of chicken non-erythroid spectrin is highly homologous to that of human red blood cell spectrin and, hence, gives further justification for its inclusion in the same protein family. The sequencing demonstrates the presence of a 106 amino acid long repeat structure, the structural motif of the human erythrocyte spectrin (Speicher and Marchesi, 1984) in the avian non-erythroid spectrin. Thus, a common structural design that was not uncovered in earlier peptide map studies is now found in two members of the spectrin family, which have, however, been shown to be clearly different by their immunological cross-reactivity (Lazarides and Nelson, 1982; Glenney and Glenney, 1983a, 1983b, 1984). This suggests a common evolutionary origin for spectrins (Glenney and Glenney, 1984; Goodman et al., 1983; Speicher and Marchesi, 1984) and that the 106 amino acid multiple repeat may be a universal structural motif of all spectrin-like proteins. The genes coding for the spectrin-like proteins may have evolved by extensive internal duplication.

Secondary structure prediction for the chicken non-erythroid spectrin reveals in each repeat three strongly predicted α-helices which are flanked by β-turns: this structural feature seems to be present also in the human erythrocyte spectrin (Speicher and Marchesi, 1984). A further analogy to the human erythrocyte spectrin is that the last α-helical span shown in Figure 5c (corresponding to the 'helix 2' in Speicher-Marchesi model) seems to be longer than the other two. Also, well-defined zones of hydrophobic and hydrophilic residues can be seen in corresponding positions in each repeat of both spectrins. These similarities suggest that a conserved folding within the repeat has been maintained despite the differences in the primary structures.

There are some distinctly well-conserved regions which correspond to the strongly predicted α-helices and β-turns (for example sequences 19 − 27 and 55 − 60). The functional significance of these regions is not clear but the characteristic structural properties common to the erythrocyte and the non-erythroid spectrins (Bennett et al., 1982; Burridge et al., 1982; Glenney et al., 1982a; Repasky et al., 1982) suggest that these may play a critical role in creating the flexible rod-like structure and allowing interaction with plasma membrane and other proteins. It may also be surmised that a well-conserved region would account for some of the immunological cross-reactivities discovered between different types of spectrin-like proteins. This possibility is also supported by the highly hydrophilic nature of this region (Westhof et al., 1984). We are currently exploring this question by raising antibodies to synthetic peptides of the sequences at the positions 55 − 60.

At this stage it is not possible to say which are the structurally unique properties underlying, for instance, the different affinities of these two proteins for other proteins such as calmodulin (Glenney and Glenney, 1984). Neither can the well-studied immunological differences be elucidated until more structural information becomes available from different spectrins and spectrin-like proteins.

Materials and methods

Screening of the cDNA library

The production and specificity of the rabbit antibodies to bovine lens spectrin-like protein p230, cross-reacting with the α-subunit of red blood cell spectrin (anti-p230 antibodies), has been described (Lehto and Virtanen, 1983; Virtanen *et al.*, 1984). Immunological screening with anti-p230 antibodies of the cDNA expression library was carried out by incubating replica filters of the library with the antibodies as described elsewhere (Helfman *et al.*, 1983, 1984). The library consisted of approximately 100 000 colonies (Helfman *et al.*, 1984).

DNA sequencing

DNA sequences were determined by the dideoxy nucleotide method (Sanger *et al.*, 1980) as modified by Biggin *et al.* (1983). The insert (1.5 kb) was released by digesting the recombinant plasmid with *Eco*RI and *Sal*I restriction endonucleases and purified by electrophoresis. It was sonicated to generate random fragments which were then cloned into M13mp8 cut with *Sma*I and treated with alkaline phosphatase (Deininger, 1983). In a parallel experiment, the insert was digested with *Alu*I, and these fragments cloned in the same vector.

Gel electrophoresis and immunoblotting

For polyacrylamide gel electrophoresis, *E. coli* DH-1 cells transformed with plasmid vector pUC8, and DH-1 cells transformed with the recombinant plasmid, were washed thoroughly and pelleted. The cells were then suspended in NaCl-P buffer (140 mM NaCl, 0.1 M phosphate buffer, pH 7.2) and sonicated. Aliquots containing equal amounts of protein were then transferred to an electrophoresis sample buffer and subjected to polyacrylamide gel electrophoresis according to Laemmli (1970).

The electrophoretically separated polypeptides were transferred for immunoblotting to nitrocellulose acetate paper (BioRad, Richmond, CA) as described by Towbin *et al.* (1979). Nitrocellulose sheets were then immersed in 3% bovine serum albumin (BSA; Sigma, St. Louis, MO) in 10 mM Tris-HCl, pH 7.2, and thereafter exposed to rabbit anti-p230 antibodies or to the rabbit pre-immune serum in 2% BSA, 0.1% Triton X-100 (BDH, Poole, UK) in 10 mM Tris-HCl, pH 7.2, for 60 min. After washing, the sheets were treated with horseradish peroxidase-conjugated swine anti-rabbit IgG (Cappel, Cochranville, PA) in 2% BSA, 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.2. The enzymatic reaction was developed with 0.03% 3,3'-diamino-benzidine-tetrahydrochloride (Fluka, Buchs, Switzerland), 0.05% H_2O_2 in NaCl-P buffer. Amido black (0.1%) was used for protein staining.

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