

https://dspace.org/

Spectrin Johannesburg: alpha and beta spectrin defects in a South African kindred with hereditary elliptocytosis

Burke, Jonathan Patrick William Graham

2014-03-20
http://hdl.handle.net10539/14248

Downloaded from a DSpace repository. https://dspace.org/

SPECTRIN JOHANNESBURG:

α AND β SPECTRIN DEFECTS IN A SOUTH AFRICAN KINDRED WITH HEREDITARY ELLIPTOCYTOSIS

JONATHAN PATRICK WILLIAM GRAHAM BURKE

SPECTRIN JOHANNESBURG: α AND β SPECTRIN DEFECTS IN A SOUTH AFRICAN KINDRED WITH HEREDITARY ELLIPTOCYTOSIS

JONATHAN PATRICK WILLIAM GRAHAM BURKE

A Thesis Submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, for the degree of Doctor of Philosophy.

Johannesburg 1998

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg, and has not been submitted for a degree in any other University.

J.P.W.G. Burke

Jonathan Patrick William Graham Burke

June 1998.

ACKNOWLEDGMENTS

I wish to express a deep debt of gratitude to Dr. Theresa Coetzer for her support, supervision and friendship throughout my PhD studies.

I also wish to thank the following people:

Dr. Stan Zail for allowing us access to his patients, for frequent blood collections, his advice and support.

The staff and students of the Department of Haematology of the University of the Witwatersrand and the South African Institute for Medical Research. In particular: Professor Barry Mendelow, Deon Van Zyl, Lesley Beeton and Rusla Lastrucci for their support and friendship.

My parents, sister Caroline and my wife Jacqueline for their unerring support and faith in me.

For financial assistance: IBM South Africa for undergraduate support, The University of the Witwatersrand, The Medical Faculty Endowment Fund, The South African Institute for Medical Research, the Foundation for Research Development, the Medical Research Council of South Africa and the Loewenstein Research Trust.

Publications

Parts of this thesis have been published in:-

Burke, J., Zail, S.S. and Coetzer, T.L. (1997) Spectrin Johannesburg: An abnormal spectrin αII domain due to partial exon skipping decreases spectrin self-association and ankyrin binding resulting in hereditary elliptocytosis. *Blood* **90**, suppl 1, 4a.

Burke, J., Zail, S.S. and Coetzer, T.L. (1998) Reduced spectrin-ankyrin binding in a South African hereditary elliptocytosis kindred homozygous for Spectrin St. Claude*. (accepted for publication June 4th 1998 by Blood)

Burke, J.P., Zail, S.S. and Coetzer, T.L. (1997) Spectrin Johannesburg: A novel α spectrin defect. Proceedings of the South African Society of Biochemistry and Molecular Biology, 14th conference, Grahamstown, p 89.

Burke, J.P., Zail, S.S. and Coetzer, T.L. (January 1997) Exon skiping caused by a stop codon. Proceedings of the South African Society of Biochemistry and Molecular Biology, 14th conference, Grahamstown, p 46.

* The mutation described by our laboratory and named Spectrin Johannesburg has since been named in the literature as Spectrin St. Claude.

Publications

Parts of this thesis have been published in:-

Burke, J., Zail, S.S. and Coetzer, T.L. (1997) Spectrin Johannesburg: An abnormal spectrin αII domain due to partial exon skipping decreases spectrin self-association and ankyrin binding resulting in hereditary elliptocytosis. *Blood* **90**, suppl 1, 4a.

Burke, J., Zail, S.S. and Coetzer, T.L. (1998) Reduced spectrin-ankyrin binding in a South African hereditary elliptocytosis kindred homozygous for Spectrin St. Claude*. (accepted for publication June 4th 1998 by Blood)

Burke, J.P., Zail, S.S. and Coetzer, T.L. (1997) Spectrin Johannesburg: A novel α spectrin defect. Proceedings of the South African Society of Biochemistry and Molecular Biology, 14th conference, Grahamstown, p 89.

Burke, J.P., Zail, S.S. and Coetzer, T.L. (January 1997) Exon skiping caused by a stop codon. Proceedings of the South African Society of Biochemistry and Molecular Biology, 14th conference, Grahamstown, p 46.

* The mutation described by our laboratory and named Spectrin Johannesburg has since been named in the literature as Spectrin St. Claude.

TABLE OF CONTENTS

1. Literature review 1

1.1 The ultrastructure of the erythrocyte membrane skeleton Error! Bookmark not defined.

1.2 The junctional complex proteins 8

1.3 Spectrin 9

1.3.1 The primary and secondary structures of α and β spectrin subunits: the spectrin 106 amino acid repeat motif 10

1.3.2 α Spectrin 15

1.3.3 β Spectrin 18

1.3.4 The origin of the spectrin 106 amino acid repeat 19

1.3.5 Spectrin tertiary and quaternary structure 20

1.3.6 Functional domains of spectrin 23

1.3.7 Spectrin heterodimer formation 23

1.3.8 Spectrin oligomers 25

1.3.9 Spectrin attachment to the lipid bilayer 27

1.3.10 Spectrin-ankyrin interactions 27

1.3.11 Spectrin-protein 4.1-adducin-actin associations 29

1.3.12 Protein 4.1-spectrin and protein 4.1-integral protein interactions 30

1.3.13 The spectrin SH3 domain 31

1.3.14 Phosphorylation 32

1.3.15 Non-erythroid spectrins 32

1.4 Biogenesis of the erythrocyte membrane skeleton 33

1.4.1 Erythrocyte differentiation models 34

1.5 Hereditary haemolytic anaemia 37

1.5.1 Forces acting on the erythrocyte membrane in the circulation 37

1.6 Aim 43

1.7 Strategy for experimental work 43

2. Protein methods 45

2.1 Materials 45

2.2 Subjects 45

2.2.1 Clinical features of probands 1 and 2 (P1 and P2) 45

2.3 Control blood samples 46

2.4 Ethical clearance 47

2.5 Hybrid spectrin (hSp) preparation 47

2.5.1 Spectrin extraction 47

4.4.1 Phosphorylation and blunt ending of PCR amplicons 131
4.4.2 Nucleotide sequencing 136

4.5 Single stranded conformational polymorphism (SSCP) analyses **136**
4.5.1 Radioactive PCR amplification of genomic DNA 136

4.6 Phenol/chloroform treatment of DNA and RNA **137**

4.7 Ethanol precipitation **137**

5. Results and discussion of DNA and mRNA analysis 139

5.1 Results **139**
5.1.1 Spectrin αII domain coding sequence 139
5.1.2 α Spectrin intron19/exon 20 boundary sequences 142
5.1.3 Investigation of DNA sequences coding for specific functional domains 145

5.2 Discussion **148**

6. Conclusion 164

7. References 166

LIST OF FIGURES

page

Figure 1. Schematic representation of a portion of the two dimensional hexagonal array of proteins that comprise the erythrocyte membrane skeleton....6

Figure 2. (A) Spectrin tryptic digest map. Two-dimensional separation, incorporating IEF and SDS PAGE, of the peptides derived from limited isotonic tryptic digestion of spectrin. (B) Linear arrangement and molecular weights of the α and β spectrin structural domains....11

Figure 3. Phasing of the three α helices that form the spectrin triple helical bundle conformational unit within the spectrin 106 amino acid homologous repeat motif sequence....14

Figure 4. Schematic representation of the relationship between the triple helical conformational repeats and functional domains of human erythrocyte spectrin....16

Figure 5. Delineation of the membrane skeleton proteins that maintain horizontal and vertical interactions in the erythrocyte membrane....40

Figure 6. Size exclusion chromatographic separation of spectrin oligomers. Shown is a typical A_{280} elution profile of spectrin oligomers from a Sepharose Cl4B column....78

Figure 7. Control spectrin subunit elution from a MonoQ ion exchange chromatography column and SDS PAGE separations of column fractions....80

Figure 8. Elution profile of P2 spectrin subunits and SDS PAGE separations of fractions....81

Figure 9. SDS PAGE gels of pooled control and P2 α spectrin fractions separated by ion exchange chromatography....83

page

Figure 10. Non-denaturing gel separations of hybrid spectrin oligomers.....84

Figure 11. Two dimensional spectrin tryptic digest peptide maps. Control, the mother's, P1 and P2 spectrin extracts were digested with trypsin under mild isotonic conditions at 0°C. Tryptic digest peptides were separated according to isoelectric point (horizontal separation) and molecular weight (vertical separation).....87

Figure 12. Two dimensional peptide maps of hybrid spectrin tryptic digests. Tryptic digests of hybrid spectrins, reassociated from control (C_α and C_β) and P1 (P_α and P_β) spectrin subunits, were separated by isoelectric focusing (horizontal) and SDS PAGE (vertical).....90

Figure 13. Two dimensional peptide maps of control and P2 α spectrin tryptic digests. Isotonic samples of control and P2 α spectrin monomers were digested with trypsin under mild conditions. Tryptic peptides were separated in two dimensions according to isoelectric point and molecular weight.....93

Figure 14. Hybrid spectrin dimer self-association assays. Hybrid spectrin dimers, formed from control (C_α and C_β) and P2 spectrin monomers (P_α and P_β), were converted to tetramers by incubation for 6 hours at 30°C. Hybrid spectrin oligomers were separated on non-denaturing gels.....96

Figure 15. SDS PAGE of hybrid spectrins used in dimer self-association assays.....97

Figure 16. Hybrid spectrin-ankyrin competitive inhibition binding assay curves. Hybrid spectrins comprising control (C_α and C_β) and P1 (P_α and P_β) spectrin monomers were assayed for their abilities to inhibit labeled control spectrin dimer binding to spectrin

page

depleted iOV....101

Figure 17. Non-denaturing gel analysis of the oligomeric states of spectrin samples used for competitive ankyrin binding assays....106

Figure 18. Control and DB spectrin-ankyrin competitive inhibition binding assay curves....109

Figure 19. Schematic representation of the tryptic cleavage sites of the α spectrin domain II 46kDa, 35kDa, 30kDa, and 25kDa peptides (represented by thin lines)....116

Figure 20. Agarose gel electrophoresis of αII domain RT-PCR amplification products. αII domain sequences coding for exons 19, 20 and 21 were amplified by RT-PCR from P1 and the mother's total reticulocyte RNA....140

Figure 21. DNA sequences of P1 spectrin αII domain 447bp and 552bp RT-PCR products....141

Figure 22. Agarose gel separation of PCR amplification products of α spectrin exon 20 sequences....143

Figure 23. Nucleotide sequence of α spectrin intron 19/exon 20 boundary in P1....144

Figure 24. Proposed mechanism for the splicing of Spectrin Johannesburg RNA transcripts and translation of the resulting mRNA....149

Figure 25. Diagrammatic representation of possible registers of the spectrin triple helical bundles α1-14 with β spectrin β11-17 and possible mechanisms for the disruption of β spectrin functions by Spectrin Johannesburg....155

Figure 26. Summary of the phenotypic effects of heterozygosity and homozygosity for the Spectrin Johannesburg ($Sp_{ЛНВ}$) genotype....160

LIST OF TABLES

page

Table 1. Components of 8% Laemmli discontinuous SDS PAGE gels used for analysing α and β spectrin monomer levels in ion exchange chromatography fractions.....55

Table 2. Components for 10% discontinuous Laemmli SDS PAGE gels for the second dimension separation of spectrin tryptic digest peptides..62

Table 3. Components for non-denaturing agarose/acrylamide gels for the analysis of the oligomeric state of spectrin samples.....64

Table 4. Components for 3.5-17% Fairbanks continuous exponential gradient gels.....72

Table 5. Relative amounts of α and β spectrin in α spectrin fractions eluted on a sodium chloride gradient from a MonoQ ion exchange chromatography column.....82

Table 6. hSp dimer to tetramer conversion assays. hSp, formed from control (C_α and C_β) and P2 (P_α and P_β) monomers, at concentrations of 0.5mg/ml and 0.3mg/ml were incubated at 30°C for 6 hours to convert hSp dimers to tetramers.....94

Table 7. Radioactivity measurements of spectrin depleted IOV bound radiolabeled control spectrin dimers from competitive ankyrin binding assays.....100

page

Table 8. Non-denaturing gel analysis of the levels of dimeric spectrin in hSp samples used for ankyrin binding assays.....105

Table 9. α/β Spectrin monomer ratios of pelleted ankyrin binding assay spectrin depleted IOV-bound spectrins.....107

Table 10. α Spectrin oligonucleotide PCR primer sequences. Sequences of primer pairs used for PCR amplification of α spectrin exons 1-8, exon 20 and αII domain cDNA sequences are given.....122

Table 11. Sequences of β spectrin PCR oligonucleotide primer pairs. Sequences of primer pairs for the PCR amplification of β spectrin exons 26, 27 and 32 are given.....123

Abstract

Spectrin is the major component of the erythrocyte protein skeleton. Spectrin tetramers, the predominant form on the membrane, consist of self-associated α/β heterodimers. β spectrin binds to the integral band 3 protein via ankyrin. Hereditary elliptocytosis (HE) is caused by skeletal protein defects that destabilise the membrane resulting in altered erythrocyte morphology. Two probands from a white South African HE kindred characterised by reduced spectrin-ankyrin binding, lowered spectrin dimer self-association and diminished spectrin in the membrane were investigated.

Control and proband monomers were isolated and reassociated into hybrid spectrins (hSp). hSp and spectrin structural analyses identified an alteration of the αII domain in the probands' spectrin between amino acids 916-1046; the mother's spectrin structure was normal. Functionally, hSp dimers reassociated with a proband α chain showed lowered self-association and reduced ankyrin binding.

RT-PCR analysis of the probands' αII domain coding sequences from reticulocyte RNA showed that they produced approximately equal amounts of two RNA messages: one with an exon 20 deletion and the other with a 12bp insertion between exons 19 and 20. The mother produced both abnormal and normal RNA messages. Genomic DNA analysis indicated that the probands are homozygous and the mother heterozygous for a T⇒G mutation 13bp upstream from the intron 19/exon 20 boundary. The mutation unmasks a cryptic 3' acceptor splice site resulting in two pre-mRNA splicing events. Firstly, 12bp of intron 19 are spliced in-frame with exon 20 introducing a translation stop codon. This message terminates translation at amino acid 935 and the peptide was not observed in membrane studies. Secondly, the presence of the stop codon causes skipping

of exon 20. Translation of this message deletes 30 amino acids in homologous repeat α9 and alters the αII domain structure. No other mutations were found and the spectrin αII domain deletion therefore has a long range influence over dimer self-association and ankyrin binding.

Ankyrin binding assays of spectrin from an individual with hereditary spherocytosis marked by a mild spectrin deficiency showed normal function. This indicated that the spectrin deficiency was not caused by a spectrin binding defect in this individual.

Abbreviations used in this text

A_{280}	Spectrophotometric absorbance value at 280nm
APS	Ammonium persulphate
bis	N,N' -methylene-bis-acrylamide
bp	base pairs
BSA	Bovine serum albumin
^{o}C	degrees centigrade
cm	centimeter
cpm	counts per minute
C-terminal	carboxyl-terminal
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
EDTA	disodium ethylene diaminetetraacetate
g	grams
g	acceleration due to gravity
HE	hereditary elliptocytosis
HPP	hereditary pyropoikilocytosis
HS	hereditary spherocytosis
hSp	hybrid spectrin(s)
IEF	Isoelectric focusing
IOV	inside out vesicles
KDS	Potassium dodecylsulphate
mA	milliampere
mg	milligram
ml	millilitre
mM	millimolar
mV	millivolt
N-terminal	amino-terminal
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecylsulphate
TEMED	N,N,N',N'tetramethylethylenediamine
TPCK	L-1-tosyl-amido-2-phenylethyl chloromethyl ketone
Tris	Tris (hydroxymethyl) aminomethane
U	unit
µCi	microcurie
µg	microgram
UK	United Kingdom
µl	microlitre
USA	United States of America
v	volume
V	volt
w	weight

The requirement for a reinforced erythrocyte plasma membrane

The distances between the cells in the tissues of large metazoan organisms and the surrounding medium are too large to rely directly upon diffusion for the supply and removal of respiratory gasses, nutrients and metabolic waste. To overcome this problem transport systems have evolved to carry respiratory gasses actively between the lungs and tissues. Specialised oxygen carrying and transfer molecules such as haemoglobin and myoglobin increase transport efficiencies. Insect tissues are supplied by an open circulatory system with a heart that pumps haemocoel around the body where it comes into contact with tissues and the respiratory surfaces of the spiracles.

Avian and mammalian species have evolved closed transport systems. In the blood of these organisms haemoglobin-filled erythrocytes are pumped in a fluid medium within closed vessels. To maximise the diffusion rates of gasses, mammalian red cells have a biconcave discoid shape that increases their surface area. Erythrocytes carry negative surface charges to stop cell aggregation thus allowing high cell densities while maintaining a free flowing blood supply. Closed circulatory systems further increase oxygen and carbon dioxide transportation by sequestering haemoglobin from bulk phase solution at haemoglobin concentrations that would produce a gelatinous mixture in bulk phase solution.

In a closed system there is no direct contact between erythrocytes and the cells of tissues. Oxygen and carbon dioxide diffuse across blood vessel walls. To bring cells

into close proximity to the blood supply, the arterial blood vessels branch repeatedly into smaller and smaller arterioles to completely penetrate the organs. Closed blood systems are therefore pressurised. Erythrocytes have to be strong and elastic to both maintain their morphology against the shearing forces in the circulation and to withstand passage through arterioles with diameters smaller than their own. In addition red blood cells must have a survival time, typically 120 days in humans, that allows the organism to balance the removal of damaged and old cells with new cell production. To maintain normal morphology under the stresses of circulation, mammalian and avian red cells have evolved a structure that reinforces the lipid membrane. This reinforcement is provided by a protein skeleton attached to the inner leaflet of the red blood cell membrane first described by Yu et al (1973). This lattice comprising several proteins (Fairbanks et al, 1971), referred to as the membrane protein skeleton, attaches to the inner leaflet of the plasma membrane.

The specific properties and high density of the erythrocyte membrane protein skeleton is a reflection of its role in maintaining erythrocyte morphology. However it is an adaptation of a more general structure for which there are homologues in a diverse range of eukaryotic cell types. The skeleton is localised in a two dimensional plane beneath the plasmalemma. In non-erythroid cells the membrane protein skeleton has a role in functions (reviewed by Carraway and Carraway, 1989) that link the plasmalemma to cytoplasmic processes and in turn to primary cellular functions such as the lateral mobility of integral proteins, the positioning of integral protein receptors,

the attachment of intracellular cytoskeletal elements that alter cell morphology and signal transduction pathways.

Mammalian erythrocytes offer an ideal experimental system for studying the membrane protein skeleton because mammalian erythrocytes are easily obtained in quantity from a range of species, are devoid in the mature stage of internal membranes and do not contain an actin based-intracellular cytoskeleton. Additionally erythrocyte lysis in hypotonic solution releases the internal components (Dodge et al, 1963) to produce ghosts that can be resealed as right-side-out (Litman et al, 1980) or inside-out-vesicles (Bennett and Stenbuck, 1979a) to allow assays of proteins on both surfaces of the membrane. The human erythrocyte membrane protein skeleton is the most extensively studied and is used as a model for other systems.

Erythrocyte membrane skeleton protein defects weaken the erythrocyte membrane causing altered red cell morphology and haemolytic anaemia. By elucidation of these defects at the molecular level it has been possible to gain insights into the normal structure and functions of the skeleton proteins and into the nature of hereditary haemolytic anaemia. Therefore in the study of the erythrocyte membrane skeleton, clinical, molecular biological and biophysical properties of the membrane protein components including their amino acid sequences, patterns of mRNA splicing and expression, recombinant peptide studies and structural analyses using tryptic cleavage, X-ray crystallography and NMR have all combined to provide a detailed picture of the structures and functions of the erythrocyte membrane skeleton.

1. Literature review

1.1 The ultrastructure of the erythrocyte membrane skeleton

Hypotonic lysis of red blood cells and subsequent removal of the haemoglobin leaves behind a cell membrane fraction referred to as erythrocyte ghosts. Solubilisation of the lipid portion of erythrocyte ghosts with non-ionic detergents (Yu et al, 1973 and Sheetz, 1979) leaves behind a protein skeleton that in the intact erythrocyte is associated with the inner leaflet of the lipid bilayer. Protein skeletons stripped of the lipid bilayer retain the same shape of the red blood cell, moreover skeletons of erythrocytes with abnormal morphology show similarly affected shape (reviewed Bennett, 1985). These observations are strong evidence for the role of the protein skeleton in the maintenance of red cell morphology. Newer data from studies of urea treated resealed erythrocyte ghosts (Khodadad et al, 1996) show that the membrane protein skeleton does not produce the morphology of a red cell per se but that it resists mechanical deformation.

Proteins of the red cell membrane skeleton separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) (Fairbanks et al, 1971) were initially named according to electrophoretic mobility. These names are listed with apparent molecular weights and alternative names in parentheses: band 1 (240kDa, α spectrin), band 2 (220kDa β spectrin), band 2.1 (200kDa, ankyrin), band 3 (diffuse 100kDa band, anion exchanger), band 4.1 (80kDa), band 4.2 (76kDa, palladin), band 4.9 (48kDa,

dematin), band 5 (43kDa, actin), band 6 (35kDa, G3PD) and band 7 (28kDa). Intact membrane skeletons left behind after Triton-X 100 solubilisation of erythrocyte lipids consist of the proteins α and β spectrin, actin, ankyrin, band 3, band 4.1, band 4.2 and band 4.9 (Yu et al, 1973). Treatment of the skeleton in 1.5M NaCl solution strips the accessory proteins (Shen et al, 1986) and leaves α and β spectrin, actin and band 4.1. These four proteins therefore define a minimum requirement for an extended skeletal network.

Initial visualisations of the membrane protein skeleton by electron microscopy were not clear due to the high density of proteins and it was not possible to ascertain separate interactions. Thus early data emanated from electron microscopy of separated proteins and biochemical analyses. However it was observed that the strength and shape of protein skeletons derived from detergent washed erythrocyte ghosts were affected by ionic strength, specific ions (Shen et al, 1986), pH and temperature (Liu et al, 1987). Skeletons could be expanded by incubation in low ionic strength buffer with dithiothreitol (Shen et al, 1986), fragmented by incubation at 37°C (Liu and Palek, 1980) or be stretched mechanically from intact cell preparations (Byers and Branton, 1985). These observations led to techniques that produced clearer electron microscope images of the positions of the proteins. Electron micrographs of treated red cell protein skeletons (Liu et al, 1987) reveal an extended two-dimensional protein array dominated by a hexagonal lattice. A schematic representation of this lattice is drawn in figure 1. Although hexagonal structures dominate the ultrastructure, some pentagons

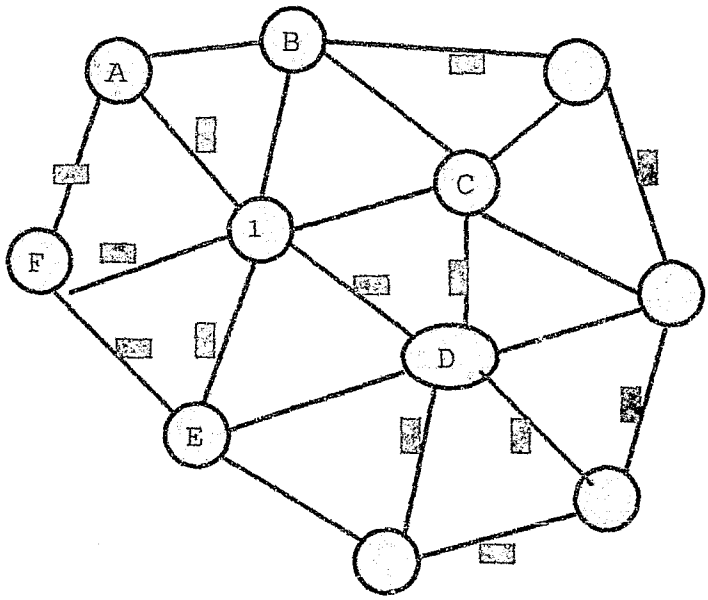


Figure 1.

Schematic representation of a portion of the two dimensional hexagonal array of proteins that comprise the erythrocyte membrane skeleton. The diagram shows a portion of the protein skeleton laminating the inner surface of the erythrocyte membrane as seen on electronmicrographs after removal of the lipid fraction of the membrane. Spectrin tetramers (green lines), associated with ankyrin (blue boxes) crosslink β-actin containing junctional complexes (filled hatched circles). β-actin, protein 4.1, tropomyosin, tropomodulin, protein 4.9 and adducin form the junctional complex. The predominant arrangement of six spectrin tetramers to each junctional complex results in an extended two-dimensional hexagonal lattice as typified by the junctional complexes A-F arranged around junctional complex 1. Adapted from Liu et al (1987).

and septagons are present (Liu et al, 1987). Linearly extended spectrin tetramers form the sides of each hexagonal structure and associate with an actin containing junctional complex at each end. Generally six spectrin tetramers associate with a short actin protofilament 30-50nm in length in the junctional complex. Spectrin tetramers were found to be the most abundant form on the membrane with evidence of hexamers and octamers. This was confirmed biochemically (Liu et al, 1984 and Morrow and Marchesi, 1981). The protein ankyrin associates with the middle of each spectrin tetramer (Liu et al, 1987). From the sizes of actin protofilaments in the junctional complexes there are predicted to be an average of 12-14 actin monomers (Liu et al, 1987 and Byers and Branton, 1985) per protofilament which was consistent with biochemical estimations (Bennett, 1989). There was also evidence for the association of actin protofilaments with other globular proteins.

Separate analyses have indicated that there are approximately 30,000 junctional complexes per erythrocyte (Pinder et al, 1978) each consisting of the proteins β-actin, tropomyosin, tropomodulin, adducin, protein 4.9 and the band 4.1a and 4.1b proteins (reviewed by Gilligan and Bennett 1993). The primary structures and functions of many of the erythrocyte membrane skeleton proteins have been elucidated. The extended protein network is attached to the lipid bilayer by spectrin. Spectrin attaches to the membrane via two integral protein interactions. The high affinity attachment is to the band 3 protein mediated by ankyrin (Bennett and Stenbuck, 1979a and b). The

second site of attachment is at the junctional complexes by an association with glycophorin C through the band 4.1 protein (Cohen and Langley, 1984).

1.2 The junctional complex proteins

The proteins of the junctional complex function to attach the tails of spectrin tetramers to the β-actin protofilament at their core. Thus it is the spectrin tetramer that crosslinks actin in the membrane. A single β actin isoform (Pinder et al, 1978) is found in erythrocytes which forms short protofilaments consisting of 12-14 monomers (Pinder et al, 1983, Byers and Branton, 1985 and Shen et al, 1986. Liu et al, 1987). The lengths of the filaments are stabilised and controlled by two molecules of tropomyosin and a single molecule of tropomodulin (Fowler, 1990). *In vitro* spectrin binds weakly to actin (Cohen and Foley, 1984) with a K_d=200μM (Ohanian et al, 1984) in a 1:1 spectrin dimer to actin monomer stoichiometry. In the junctional complexes there is a stoichiometry of 1 spectrin dimer bound per 2-3 actin monomers. Thus six spectrins can attach at a junctional complex to produce the hexagonal array of the protein skeleton. Binding of the band 4.1 protein to spectrin increases its affinity for actin (Cohen and Langley, 1984). The assembly of the hexagonal structures incorporating spectrin and actin in the junctional complexes is postulated to be under the control of the protein adducin (Mische et al, 1987 and Hanspal and Palek, 1987) which is expressed early in erythropoiesis (Nehls et al, 1991).

The band 4.9 protein, also known as dematin, has an actin-bundling function (Siegel and Branton, 1985) that is abolished by phosphorylation (Husain-Chisti et al, 1988 and 1989). The function of actin-bundling and the relevance of the modulation of this activity by phosphorylation is not known. The initial band 4.9 preparations contained three proteins with molecular weights of 48kDa, 52kDa and 55kDa as determined by SDS PAGE. The first two peptides are related in structure with actin-bundling activity and have been called dematin (Husain-Chisti et al, 1989). The palmitoylated 55kDa peptide (Ruff et al, 1991) has properties consistent with a membrane-associated guanylate kinase; a family of proteins that are involved in signal transduction pathways.

1.3 Spectrin

Spectrin is the backbone of the erythrocyte membrane protein skeleton and is calculated to account for 50% (Sheetz, 1979) to 75% (Yu et al, 1973) of the protein mass depending upon the type of measurements used. The name spectrin is derived from the word spectre (Marchesi and Steers, 1968) and the fact that it was originally purified from red blood cell ghosts. Spectrin tetramers are the agents that both crosslink actin in the junctional complexes to produce an extended network and attach the skeleton to the membrane. Thus protein skeleton properties are in large part attributable to the characteristics of spectrin.

Measurements from electron microscopy show that dimeric spectrin forms an elongated rod-like structure with a contour length of approximately 1000Å and width of 50Å (Shotton et al, 1979); the length is doubled in the tetrameric form. Spectrin dimers comprise an α and β subunit with molecular weights of 240kDa and 220kDa respectively as determined by SDS PAGE (Fairbanks et al, 1971). The subunits associate in an extended antiparallel fashion along their lengths in the dimer to form the elongated structures seen in electronmicrographs (Shotton et al, 1979).

The structure and functions of human spectrin have been the subjects of intensive investigations. Additional to this has been the parallel study of non-erythroid spectrins referred to as fodrins (Winkelmann and Forget, 1993) and the spectrin superfamily of proteins.

1.3.1 The primary and secondary structures of α and β spectrin subunits: the spectrin 106 amino acid repeat motif

Erythrocyte spectrin consists of two non-identical α and β subunits. Two dimensional mapping, incorporating isoelectric focusing (IEF) and SDS PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis), of mild isotonic tryptic spectrin digest peptides (Speicher et al, 1980, Speicher and Marchesi, 1982) produces a series of reproducible peptides. The peptides represent five α spectrin and four β spectrin structural domains shown in figure 2 with their linear alignments. Spectrin domains αI to αV are named from the N-terminal with molecular weights deduced from SDS

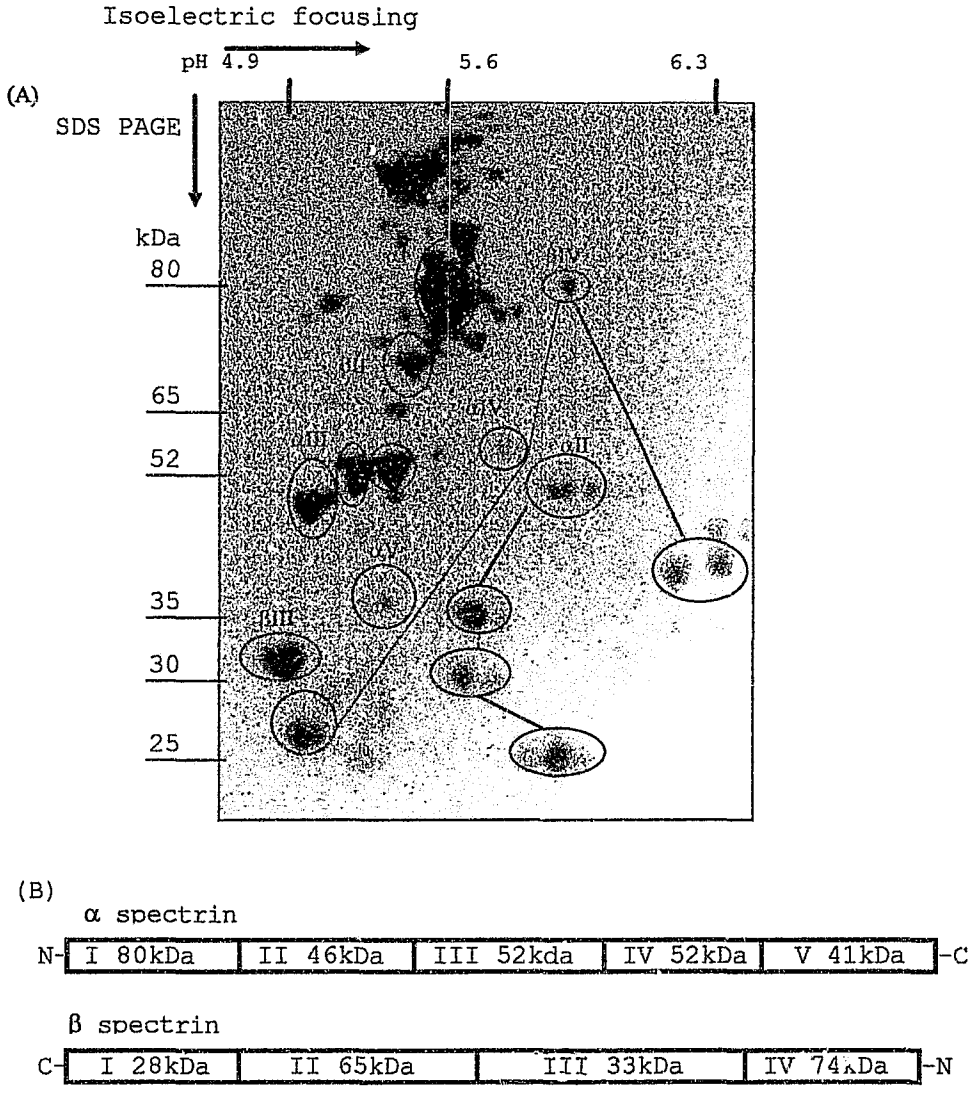


Figure 2

(A) Spectrin tryptic digest map. Two-dimensional separation, incorporating IEF and SDS PAGE, of the peptides derived from limited isotonic tryptic digestion of spectrin produces a reproducible series of five α spectrin (I-V outlined in red) and four β spectrin (I-IV outlined in green) structural domains and their breakdown products.

(B) Linear arrangement and molecular weights of the α and β spectrin structural domains shown in (A).

PAGE of: 80kDa, 46kDa, 52kDa, 52kDa and 30kDa (Speicher et al, 1982) respectively. β Spectrin domains βI to βIV are named from the C-terminal with molecular weights of: 28kDa, 65Da, 33kda and 74kDa respectively. These structural domains have varying degrees of resistance to tryptic cleavage and the resulting breakdown peptides have been identified by peptide mapping (Speicher et al, 1982 and Knowles and Bologna, 1983).

The existence of spectrin's structural domains coupled with evidence from circular dichroism showing a high α-helical content (Ralston, 1978 and Calvert et al, 1980b) and data indicating that spectrin contained independently mobile segments (Calvert et al, 1980b) suggested an early structural model (Speicher et al, 1982). In this model, triple α-helical domains were joined together by proteolytically sensitive hinges. The triple helix was introduced because it was consistent with the measured length of spectrin which cannot accommodate a single extended helix. This early model was revised after partial amino acid sequencing of both subunits showed that spectrin's primary structure can be divided into homologous 106 amino acid repeat sequences (Speicher and Marchesi, 1984). In this revised model the 106 amino acid repeats form triple helical structural units of approximate molecular weight 12kDa joined together by short non-helical regions. Thus spectrin was considered essentially to be a chain of repeating triple helical bundles strung together in a linear fashion. The repeat sequences are characterised by an invariant tryptophan at position 45 and a highly conserved leucine at position 26. There are in addition positions with a strong bias for

one amino acid or a conservative substitution of the preferred residue. These positions are listed with the amino acids in parenthesis using the single letter amino acid code: 1(I), 12(W), 15(L), 22(R), 35(F) 46(I), 71(L), 38(D), 41(D), 48(D or E), 72 (H) and 101 (H). The full amino acid sequences of α (Sahr et al, 1990) and β spectrin (Winkelmann et al, 1990), deduced from the cDNA sequences, confirmed that both molecules are largely formed from homologous repeats.

Within a 106 amino acid repeat motif, the first helix of a triple helical bundle conformational unit in the original predicted structure began at position 35 (Speicher and Marchesi, 1984). Deletion analysis of recombinant peptides of *Drosophila* α spectrin repeats 14 and 15 (Winograd et al, 1991), referred to as α14 and α15, identified the stable conformational unit as starting between the consensus amino acid leucine at position 26 and position 30. The relationship between 106 amino acid repeats and the triple helical bundle conformation is shown in figure 3.

The X-ray crystal structure of recombinant *Drosophila* α spectrin (Yan et al, 1993) expressed from asparagine 29 in α14 to aspartic acid 28 in repeat α 15 confirmed the existence of the predicted triple helical structure. In this structure, the three helices in the triple helical bundle are packed tightly together. Comparison of the sequences of other spectrin 106 amino acid repeats with the *Drosophila* repeat shows that amino acids involved in important interactions in the triple helical bundle are conserved. Thus the triple helix structure found in *Drosophila* spectrin appears to represent a general case for other spectrins. This has been confirmed by an NMR derived structure for

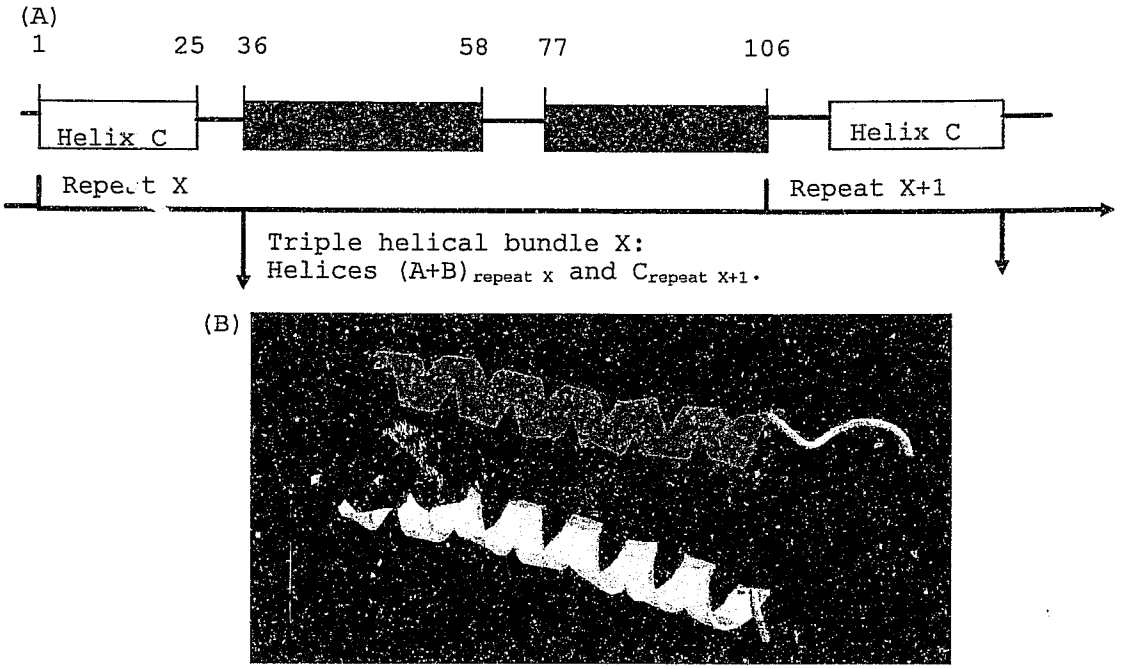


Figure 3.

Phasing of the three α helices that form the spectrin triple helical bundle conformational unit within the spectrin 106 amino acid homologous repeat motif sequence. Diagram A shows the amino acids within the 106 amino acids of a homologous repeat motif X that are predicted to form α helices (depicted by coloured boxes) and turns (black lines). The numbers refer to the position of an amino acid within the 106 amino acid repeat defined by Speicher and Marchesi (1984).

α Helices A and B of repeat X and helix C of repeat X+1 are predicted to form a triple helical bundle conformational unit. Diagram (B) shows the NMR derived structure of a triple helical bundle of a chicken α spectrin repeat motif (Pascual et al, 1997) drawn with Raswin from co-ordinates taken from the Brookhaven Protein Data Bank. The exact phasing of the three helices within any bundle depends upon the particular repeat but the model shown is predicted to be similar for all repeats.

chicken brain α spectrin repeat 16 (Pascual et al, 1996 and Pascual et al, 1997) shown in figure 3(B). Recombinant peptide studies of the human repeat α1 (DeSilva et al, 1997) indicate that the phasing of the human 106 amino acid repeat is similar to that for *Drosophila* repeat α14. The stability of expressed peptides was greatly influenced by the addition of two amino acids to the C-terminal of the peptide.

Spectrin repeat motifs and related homologues have been found in a number of actin cross-linking molecules including dystrophin and α actinin (Parry et al, 1992). The presence of spectrin-like motifs in these proteins places them into the spectrin superfamily.

1.3.2 α Spectrin

Primary structure

Human erythrocyte α spectrin consists of a 2429 amino acid polypeptide chain (Sahr et al, 1990) with a calculated molecular weight of 280kDa; larger than the 240kDa deduced from SDS PAGE (Fairbanks et al, 1971). Dominating the α spectrin amino acid sequence, as shown in figure 4, are 20 segments with homology to the 106 amino acid repeat. Alignment by either leaving gaps or adding amino acids to the α spectrin homologous repeats (Sahr et al, 1990) shows that repeats 1-9 and 12-19 are the most closely related with the highest degree of conservation at positions 15(L), 26(L), 35(F), 45(W), 46(I), 68(L). 11 of the repeats are 106 amino acids long, repeats 1, 6, 12 and 14 can be aligned with a single gap, repeats 11, 18, 19, 20 and 21 are 102, 109,

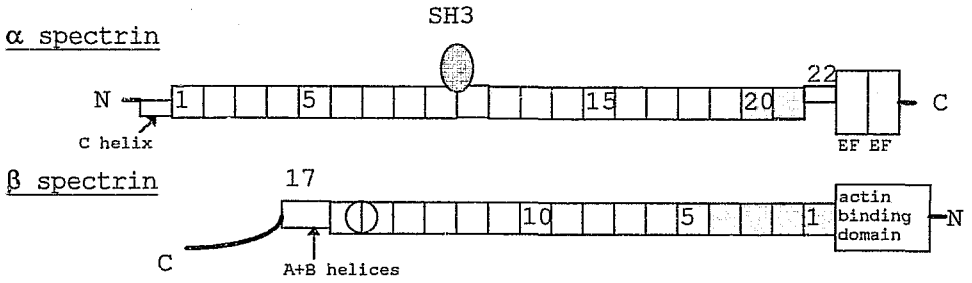


Figure 4.

Schematic representation of the relationship between the triple helical conformational repeats and functional domains of human erythrocyte spectrin. Shown is a schematic representation of the human erythroid spectrin subunits which comprise of tandemly repeated triple helical segments (squares) that spatially separate specific functional domains. α Spectrin segment 10 forms an SH3 domain (filled loop), repeats 20 and 21 are essential dimer nucleation sites (hatched) and the C-terminal domain has homology to EF hand calcium binding motifs (EF). The β spectrin C-terminal domain is the site of phosphorylation (curved line), sequences within repeats 15 and 16 form the minimum requirements for the ankyrin binding domain (circle), repeats 1-4 form the essential dimer nucleation domain (hatched) and the N-terminus is the actin binding site.

The α spectrin homologous repeat α1 produces an unmatched C helix and β spectrin repeat β17 produces an incomplete A+B helix structure. Spectrin dimers self-associate by a reciprocal interaction whereby the unpartnered helices from each dimer form complete triple helical bundles. (Diagram adapted from Speicher et al, 1992 and DeSilva et al, 1997).

107, 114 and 111 amino acids long respectively. The N-terminal 22 amino acids and the first 50 amino acids after segment 21 form partial repeats. The sequence of 77 amino acids of segment 10 is atypical and does not conform to the spectrin repeat motif. This sequence is homologous to the *src* homology domain 3 (SH3) in the *src* non-receptor cytoplasmic tyrosine kinase family of proteins (Sahr et al, 1990). SH3 domains are found in a variety of cytoplasmic proteins and are thought to act as a means of targeting proteins to a particular cellular location. The SH3 domain is highly conserved between human α spectrin and chicken α fodrin (Wasenius et al, 1989). Spectrin may function in anchoring *src* tyrosine kinase substrates at the plasma membrane. The C-terminal 100 amino acid sequence has homology to calcium binding EF hands (Sahr et al, 1990). The significance of calcium binding is not understood and this domain may be vestigial.

α Spectrin gene and mRNA

The α spectrin gene on chromosome 1 (Huebner et al, 1985) contains 52 exons (Kotula et al, 1991). The 3' acceptor splice sites of intron/exon boundaries are all defined by the invariant -AG- consensus sequence (Padgett et al, 1986) preceded by a polypyrimidine tract at most of the boundaries. The 5'donor splice sites all have the invariant -GT- and consensus donor sequence with the exception of intron 32 where the invariant -GT- is replaced by a -GC-.

An 8kb α spectrin mRNA is transcribed (Sahr et al, 1990) from the α spectrin gene. At the 5' and 3' terminii respectively, the transcript contains 187bp and a 528bp untranslated regions on each side of a single 7287bp open reading frame.

1.3.3 β Spectrin

Primary structure

β spectrin is formed from 2137 amino acids (Winkelmann et al, 1990) with a calculated molecular weight of 246kDa, greater than the 220kDa measured from SDS PAGE electrophoretic mobility (Fairbanks et al, 1971). The β spectrin amino acid sequence can be divided up into three domains (Winkelmann et al, 1990) distinct from the structural domains produced by tryptic digestion. Domain II is a series of 17 segments each with varying degrees of homology to the spectrin repeat motif. Domains I and III have no homology to the spectrin repeat structure. The structural and functional domains of β spectrin are shown in figure 4.

The N-terminal 272 amino acid domain I shows a high degree of conservation with the actin binding domains of α-actinin and dystrophin (Winkelmann et al, 1990).

The 17 segments that predominate β spectrin structure have homology to the 106 amino acid repeat motif (Winkelmann et al, 1990) with a high degree of conservation of the consensus leucine and tryptophan residues at positions 26 and 45 respectively. Repeats 1 and 2 have 6 and eight additional amino acids and repeat 112 is 99 amino acids long. The sequences of repeats 1 and 15 diverge from the defined repeat sequence. Repeat 15

contains a partial repeat sequence between amino acids 1-47. Positions 48-106 are atypical and form part of the ankyrin binding domain with repeat 16 (Kennedy et al, 1991).

β Spectrin domain III is a 52 amino acid sequence with no homology to the spectrin repeat and codes for a caesin kinase LSSSWE phosphorylation site (given in single letter amino acid code) (Winkelmann et al, 1988).

β Spectrin gene and mRNA

The β spectrin gene, located on chromosome 14 (Prchal et al, 1986 and Fukushima et al, 1990) contains 32 exons (Amin et al, 1993). The exon-intron boundaries conform to the splice site consensus sequences (Padgett et al, 1986) except for exon 20 where the normal 5' donor -GT- site is replaced with -GC-.

Transcription of the β Spectrin gene gives rise to a 7.5kb and a 9 kb (Prchal et al, 1987) RNA message. There is a single 6411bp open reading frame (Amin et al, 1993) that codes for 2137 amino acids. The reason for the two transcripts remains unknown.

1.3.4 The origin of the spectrin 106 amino acid repeat

To explain the existence of the tandemly repeated motifs found in the spectrin superfamily, it has been hypothesised that they were derived during evolution by duplication of an ancestral gene. This hypothesis predicts a correlation between the exonic organisation and the 106 amino acid motif (Byers et al, 1992). The arrangements of exons in the α and β spectrin genes do not lend support to this hypothesis as there is no obvious coincidence (Kotula et al, 1991 and Amin et al, 1993). This does not however discount the

theory as the modern position of the exon boundaries may be the result of selective pressures that forced divergence of their initial positions that now obscures the evidence of the original duplication events.

1.3.5 Spectrin tertiary and quaternary structure

The three α helices formed by the spectrin conformational unit pack closely together and interact in a highly specific manner (Yan et al, 1993). The triple helical bundles are joined by hinge regions. Thus the tandem repetition of the motif structures along each monomer (Sahr et al, 1990 and Winkelmann et al, 1990), a picture of spectrin is presented of a linear molecule in which the repeat units provide lateral strength and spatially separate functional domains as shown diagrammatically in figure 4. However mounting evidence indicates that this model is inadequate and that the hinge regions are critical to the properties of spectrin. This is deduced from observations that the properties of recombinant peptides incorporating small numbers of repeat units do not reflect the properties of whole spectrin (reviewed by Speicher and Ursitti, 1994).

Electron microscopic (Shotton et al, 1979) and biophysical evidence (Calvert et al, 1980a) show that the α and β spectrin subunits intertwine to form a non-covalently associated heterodimeric noodle-like quaternary structure. The separate subunits retain spectrin like properties and thus appear to be able to fold independently of each other.

Relationship between the biophysical properties of the spectrin repeat motif and the properties of whole spectrin

The human erythrocyte spectrin dimer is a long, rod-like, flexible structure. Each monomer's primary structure is dominated by tandemly repeated structural motifs. Logically it would seem therefore that spectrin's properties are in large part contributed by the 106 amino acid motifs. While many of the properties of the repeat motif are coincident with those of whole spectrin, there are also important anomalies to be addressed.

Many of the biophysical properties of a recombinant peptide incorporating the first structural repeat unit of human α spectrin are similar to those of whole spectrin (DeSilva et al, 1997). However it was observed that in low ionic strength solution there was little alteration in the tertiary structure of the peptide or the hydrodynamic volume. Thus the triple helical bundle remained stable and did not refold into a more extended structure such as a single helix. Therefore this does not account for the ability of spectrin to extend in low ionic strength solution or for its flexibility.

The addition of 106 amino acid repeat units to recombinant spectrin peptides does not result in a linear increase in the Stokes radius of the peptide (Ursitti et al, 1996). However increasing the number of repeat units increases the whole spectrin-like properties of a recombinant peptide (Menhart et al, 1996). Therefore the connections between the helical repeats do not simply extend the molecule in a linear fashion. There are also circular dichroism data showing that spectrin contains more β sheet (LaBrake et al, 1993) than would be predicted from current structural models. These properties thus result from the hinge regions joining the triple helical bundles.

The recombinant *Drosophila* repeat α14 peptide (Yan et al, 1993) used in X-ray crystallographic studies crystallised as a dimer. A model of the structure of the hinge regions that join two repeats was proposed. In this model, the triple helix bundles are joined by an extension of the C helix of segment X that is made continuous with the A helix of segment x+1 (Yan et al, 1993). This implies that structural units are added in a linear fashion without any obvious mechanism for flexibility. Hinge region structure thus needs to be revised and included into a model of the repeat structural unit to explain the extensibility and flexibility of whole-spectrin.

A second reported property of spectrin and fodrins is the ability of allosteric effects to be transmitted along the length of the molecule. This has been well documented in two cases. In the first case, bovine brain spectrin studies (Harris and Morrow, 1990 and Hu and Bennett, 1991) have demonstrated that a structural change introduced into the middle of the α subunit by proteolysis is transmitted to a terminal functional domain of β spectrin. This also demonstrates the existence of inter-chain interactions. In the second case, erythrocyte spectrin tetramers *in vitro* bind ankyrin with higher affinity than dimers (Cianci et al, 1988). This demonstrates the transmission of an allosteric effect between the self-association site to the ankyrin binding site. The transmission of allosteric effects must be mediated by the hinge regions between the repeat motifs which pass on conformational strain between triple helical motifs. Each repeat is therefore not a completely independently mobile unit as initially indicated (Calvert et

al, 1980b). A comprehensive spectrin model thus also needs to explain details the communication between the helical bundles.

1.3.6 Functional domains of spectrin

The two principle functions of spectrin are to crosslink actin protofilaments in the junctional complexes thus forming a two dimensional protein skeleton and to attach the protein array to the membrane lipid bilayer. Spectrin heterodimers are monovalent for actin and form divalent spectrin tetramers which predominate on the membrane. Spectrin is then attached to the membrane via associations with integral proteins. Other functions of spectrin are associated with calcium binding activity and phosphorylation. The function of the α spectrin SH3 domain is undetermined.

1.3.7 Spectrin heterodimer formation

α and β spectrin chains associate through non-covalent interactions in an antiparallel fashion to form an extended heterodimer (Calvert et al, 1980a) with a binding stoichiometry of 1α:1β (Yoshino and Marchesi, 1985). *In vitro* side-by-side association occurs within seconds and is specifically initiated at nucleation sites on the subunits towards the actin binding region (Speicher et al, 1992). The α spectrin nucleation site is situated in homologous repeats 19-22 within the αV domain; the β spectrin site is in repeats 1-4 (Ursitti et al, 1996) at the N-terminal of the βIV domain. These nucleation sites are essential as heterodimer formation does not occur if they are

deleted. Additionally side-by-side subunit pairing is terminated at points of tryptic cleavage.

Three of the homologous repeats within the essential nucleation sites contain additional amino acid sequences given in the single letter amino acid code in parentheses: α20 (LEKQLPLQ), α21 (QKEERARQV) (Sahr et al 1990) and β2 (KLEQLARR) (Winkelmann et al, 1990). The sequences are all inserted in similar positions close to amino acid 30 in the homologous repeat structure which would place before helix A in the triple helical bundle (Yan et al, 1993). It seems likely that the structures of the nucleation sites in α and β spectrin (Speicher et al, 1992) are complementary and directly interact with each other. This implies that the two chains in a heterodimer are forced to associate in a specific manner that will affect the register of the homologous repeat units relative to one another along the entire length of the dimer. The requirement for specific nucleation sites for subunit interaction explains the finding that subunits in reconstituted dimers (Yoshino and Marchesi, 1984) always have similar registers with respect to one another when viewed by electron microscopy.

Side-by-side associations and interactions of spectrin subunits along the length of the heterodimer

Studies of spectrin tryptic digest peptides (Morrow et al, 1980) indicate an association between a β spectrin 65kDa C-terminal peptide and the 80kDa αI domain. The 65kDa β spectrin peptide incorporates the ankyrin binding function of spectrin and could be cleaved

into a 52kDa and a 28kda peptide. Size exclusion chromatography of tryptic digests of spectrin dimers produced a peak with a retention time similar to the spectrin dimer (Speicher et al, 1992). The C-terminal β spectrin domain dissociates from the digested dimer and the 80kDa αI peptide partially dissociates. Thus cleaved subunits maintain extensive pre-existing inter-chain non-covalent interactions along the length of a heterodimer. Apart from the nucleation sites (Speicher et al, 1992), such interactions are not well characterised.

Side-by-side non-covalent interactions within the spectrin heterodimer allow for inter-chain transmission of structural perturbations. Structural perturbations can result from allosteric effectors, mutations, proteolysis or phosphorylation. This is important in light of the evidence for allosteric transmission along the length of spectrin subunits (Cianci et al, 1988, Hu and Bennett, 1991). This has been particularly well demonstrated for bovine brain spectrin which is a close homologue of erythroid spectrin (Harris and Morrow, 1990). In the study, the binding of calmodulin to the central part of the α subunit allowed a calcium dependent protease to cleave the β subunit at the C-terminal. The β subunit is not efficiently cleaved in the absence of calmodulin binding. Thus the effect of the calmodulin binding was transmitted both along the spectrin dimer and between the monomers.

1.3.8 Spectrin oligomers

Spectrin dimers are monovalent for actin and thus cannot crosslink the junctional complexes in the membrane. Spectrin tetramers crosslink actin and therefore dimer

self-association is a central function for the stabilisation of the membrane protein skeleton (Liu and Palek, 1980). Spectrin tetramers are the predominant species on the erythrocyte membrane (Liu and Palek, 1980) however higher order oligomers also occur (Morrow and Marchesi, 1981).

Spectrin tetramer formation

Spectrin dimer self-association into tetramers is a reversible process governed by a thermodynamic equilibrium (Ungewickell and Gratzer, 1978). Tetramer formation is inhibited in low ionic strength solution but is unaffected by the state of β spectrin phosphorylation. The phasing of the triple helical conformational unit in the homologous repeat structure leaves unpartnered α helices at the N-terminus of α spectrin and C-terminus of β spectrin repeat 17 as shown in figure 4. Spectrin dimers self-associate by non-covalent interactions between these unpartnered α helices. The N-terminal helix C of the α spectrin in the first dimer (DeSilva et al, 1992, Speicher et al, 1993 and Kotula et al, 1993) interacts with helices A and B of homologous repeat 17 of β spectrin in the second dimer (Tse et al, 1990 and Kennedy et al, 1994). This interaction is reciprocated by the second dimer and is referred to as head-to-head. In the proposed model of dimer interaction, the α and β subunit associations combine the partial motifs into complete triple helical bundles.

Spectrin dimer to tetramer interconversion is slow below 30°C (Ungewickell and Gratzer, 1978). This is caused by the energy of activation required to convert spectrin dimers from a closed conformation unable to form tetramers to an open conformation (Morris and

Ralston, 1989). A mechanism for this has been proposed (Speicher et al, 1993) whereby the self-association sites of α and β spectrin interact within the heterodimer in a similar manner to the formation of a tetramer. Thus the N-terminal of α spectrin is looped back to interact with the C-terminal of the β spectrin forming a closed hairpin loop conformation. Raising the temperature supplies enough energy to break this interaction leaving the dimer self-association site open to link with a second open dimer.

Formation of higher order spectrin oligomers

Spectrin dimers can sequentially interact to produce hexamers and higher order oligomers (Morrow and Marchesi, 1981). The mechanism is similar to that for tetramer formation. After extraction, *In vitro* spectrin concentrations are typically maximal at 4-5 mg/ml which is too low for significant formation of higher order oligomers. The restriction of spectrin to a narrow two-dimensional shell below the membrane *in vivo* increases the effective concentration of spectrin to 200mg/ml (Lux, 1979) and higher oligomer formation.

1.3.9 Spectrin attachment to the lipid bilayer

Spectrin is tethered to the erythrocyte membrane through interactions with the integral proteins band 3 via ankyrin and glycophorin C via the band 4.1 protein.

1.3.10 Spectrin-ankyrin interactions

The spectrin-ankyrin binding site

Spectrin selectively associates with the inner leaflet of the red cell membrane (Bennett and Branton, 1977 and Litman et al, 1980). This specificity is the result of an interaction with the protein ankyrin (Luna et al, 1979 and Bennett and Stenbuck, 1979a). Ankyrin interacts with the integral band 3 protein thus tethering spectrin to the membrane (Bennett and Stenbuck, 1979b).

The spectrin-ankyrin binding site resides towards the C-terminal of β spectrin (Morrow et al, 1980). Deletion analysis of recombinant β spectrin peptides identified the minimum ankyrin binding site between amino acids 1,768-1,898 (Kennedy et al, 1991). This region encompasses homologous repeat 15 and 24 amino acids of repeat 16. The 15th repeat is atypical and lacks the invariant tryptophan at position 45. It also contains a non-homologous 43 amino acid sequence in the second half of the repeat that is highly conserved between erythroid β spectrin and β fodrin.

On the erythrocyte membrane ankyrin binds to spectrin tetramers with a 1:1 stoichiometry (Goodman and Weidner, 1980 and Yu and Goodman, 1979) despite there being two binding sites per tetramer. Contradictory data exist describing the affinity of ankyrin for dimeric and tetrameric spectrin. Goodman and Weidner (1980) found no difference in bioassays using spectrin depleted inside out vesicles (IOV); whereas Cianci et al (1988), Weaver et al (1984) and Lu et al (1985) reported higher affinities of ankyrin for spectrin tetramers *in vitro*. Phosphorylation of spectrin does not affect ankyrin binding.

Ankyrin

Human erythrocyte ankyrin is part of a class of proteins also referred to as syndeins (reviewed by Peters and Lux, 1993). The major component is ankyrin or protein 2.1 with a calculated molecular weight of 206kDa (Lambert et al, 1990). Other syndeins are thought to be isoforms that arise by differential splicing of a single ankyrin RNA transcript (Lux et al, 1990).

Chymotrypsin cleaves ankyrin into three functional domains (Peters and Lux, 1993): an N-terminal 89kDa peptide with band 3 and tubulin binding activity, a 62kDa peptide that binds spectrin and intermediate filament proteins and a C-terminal peptide that modulates spectrin and band 3 binding.

The formation of the spectrin, ankyrin, band 3 ternary complex is regulated by ankyrin phosphorylation (Cianci et al, 1988 and Lu et al, 1985). Ankyrin is reported to have greater affinity for spectrin tetramers than dimers *in vitro* (Weaver et al, 1984 and Cianci et al, 1988) which is reversed upon phosphorylation.

1.3.11 Spectrin-protein 4.1-adducin-actin associations

Spectrin tetramers crosslink actin in the junctional complexes. Spectrin binds actin with low affinity (Cohen and Langley, 1984) at a site located towards the N-terminal of the β spectrin chain (Becker et al, 1990) within the first 422 amino acids (Li and Bennett, 1996). This region includes the α-actinin homology domain (Sahr et al ,1990). The affinity of spectrin for actin is increased approximately 100 fold by the association of the band 4.1 protein with a heterodimer (Cohen and Langley, 1984). Protein 4.1 does not influence the

actin binding of β spectrin alone implying that the combination of the subunits supplies full binding activity. Recombinant peptide studies (Li and Bennett, 1996) show that full actin binding is achieved with the association an α spectrin helical repeat domain with β spectrin and a stable adducin-spectrin-actin ternary complex requires β spectrin peptides containing the first two helical domains. The model proposed from these results places the end of an actin filament, capped with adducin, at the N-terminal of β spectrin.

Protein 4.1 associates with the integral protein glycophorin C (Conboy, 1993) and is a low affinity tether for spectrin to the membrane.

1.3.12 Protein 4.1-spectrin and protein 4.1-integral protein interactions

SDS PAGE of erythrocyte membrane proteins resolves protein 4.1 into two bands, 4.1 a and b, at 80kDa and 78kDa respectively. The deamidated a form accumulates with erythrocyte age (Mueller et al, 1987) and the increased a:b ratios measured by scanning denistometry of SDS PAGE gels are used as an index of the age of a population of erythrocytes.

Proteolytic cleavage of protein 4.1 results in four structural domains of molecular weights: 30kDa, 16kDa, 10kDa and 22-24kDa domains (Leto and Marhesi, 1984). The C-terminal domain contains the deamidation site and thus has two molecular weights estimated from SDS PAGE.

Functions associated with the band 4.1 structural domains have been elucidated (reviewed by Conboy, 1993): The 30kDa N-terminal domain is thought to contain

glycophorin A, glycophorin C, band 3 protein and calmodulin binding sites. Phosphorylation of the proline rich 16kDa domain modulates the formation of the ternary complex between protein 4.1, band 3 and spectrin. The 10kDa domain binds spectrin and promotes ternary complex formation between protein 4.1, actin and spectrin (Correas et al, 1986). The C-terminal 22-24kDa domain contains the asparagine residue 502 that is deamidated and therefore has two electrophoretic mobilities on SDS PAGE. There are no known functions for this domain.

1.3.13 The spectrin SH3 domain

α Spectrin segment 10 is not homologous to the spectrin repeat structure. Instead it is an SH3domain (Sahr et al, 1990). SH3 domains recognise proline rich SH3 binding sequences of about 10 amino acids in length (Ren et al, 1993) that contain a consensus PxxP motif (Williamson, 1994). An NMR structure of a chicken brain α spectrin SH3 domain has been elucidated (Blanco et al, 1997). The homology of human erythrocyte and chicken brain spectrins (Wasenius et al, 1989) has lead to a model that fits the SH3 domain between helices B of repeat α9 and helix C of repeat α11 domain (Fournier et al, 1997) as shown in figure 4. The N- and C-termini of the SH3 domain are in close spatial proximity and there is no disruption of the triple helical structural units. Thus helices A9 and B9 form a complete triple helical bundle with C11.

SH3 domain carrying proteins include the protein Grb2 which complexes with the GTP-GDP exchange Sos protein (Buday and Downward, 1993) in the Ras signalling

pathway and the Abl family of non-receptor phosphotyrosine kinases (reviewed by Feller et al, 1994). The function of the SH3 domain of human erythrocyte spectrin is not known.

1.3.14 Phosphorylation

Incubation of intact erythrocytes with radiolabelled phosphate has shown that each spectrin dimer is phosphorylated four times (Harris et al, 1980 and Harris and Lux, 1980). The phosphorylation sites are clustered in the C-terminal domain III of β spectrin. The physiological purpose of erythrocyte spectrin phosphorylation is not known but may regulate the interactions of the subunits in a heterodimer (Mische and Morrow, 1989).

1.3.15 Non-erythroid spectrins

Just as the human erythrocyte protein membrane skeleton is used to model homologous structures in other cell types and species, the structure of erythroid spectrin is a model for a family of spectrin-like proteins. This family is defined by the existence of homologues to the spectrin 106 amino acid repeat motif. Included are the differentially spliced products of the four human spectrin genes that incorporate the erythroid and non-erythroid spectrins referred to as fodrins. The extended spectrin superfamily also includes α actinin and dystrophin and more distantly related proteins (reviewed by Gallagher and Forget, 1993 and Winkelmann and Forget, 1993).

Functions of non-erythroid spectrin molecules

Spectrin-like molecules all function to associate cortical actin crosslinking networks to the plasmalemma (Winkelmann and Forget, 1993 and Lodish et al, 1995). Most crosslinked actin networks are not as simple as that found in erythrocytes and incorporate longer actin filaments and tubules. Spectrin-like molecules are thus involved in the control of integral protein lateral mobility and membrane location as well as cell plasmalemma morphological changes (Carraway and Carraway, 1989). These roles are critical to cellular responses to intra and extracellular stimuli.

1.4 Biogenesis of the erythrocyte membrane skeleton

The biogenesis of the membrane protein skeleton is part of the study of protein ultrastructure assembly. The diversity of eukaryotic cell morphologies is directed and maintained by the specificity of cytoskeletal structures consisting of actin filamets, microtubules and intermediate filaments that self-assemble during differentiation. However the complexity of ultrastructures are such that there has to be control exerted over the protein components to ensure correct stoichiometric and spatial assembly. Control is exerted over: the modulation of the specificity of component interactions, the temporal availability of components during differentiation, the spatial availability of components and the provision of pre-existing template structures for the deposition of components.

The relatively simple and well characterised mammalian erythrocyte membrane skeleton offers a model system for the study of these mechanisms (Lazarides, 1987).

However because mammalian erythrocytes are anucleate and lose microtubules and intermediate filaments during maturation these structures have been studied in chicken erythrocytes.

1.4.1 Erythrocyte differentiation models

Erythropoiesis falls within the larger subject of eukaryotic cell differentiation. Erythropoiesis occurs in bone marrow and is the process that results in the differentiation of a pluripotential stem cell into a mature erythrocyte. In mammalian and avian species a pluripotential stem cell is committed to the erythrocyte lineage by a pathway during two replicative progenitor stages called the burst-forming unit (BFU-e) and the colony forming unit (CFU-e) before terminal differentiation into a mature erythrocyte (Till and McCulloch, 1980).

Stage specific cells cannot be isolated from bone marrow for erythropoietic studies and transformed cultured cell lines arrested at particular stages of differentiation are used to model membrane skeleton biogenesis. Avian erythroblastosis and S13 virus transformed chicken cell lines (Woods et al, 1986) and Rauscher erythroleukaemia virus-transformed murine cells (MEL cells) have provided useful models for the early stages of non-mammalian and mammalian erythropoiesis respectively (Hanspal et al, 1992b). Transformed cell lines do not respond to the hormone erythropoietin and have to be induced chemically into partial maturity for the study of later stages of the differentiation process (reviewed by Hanspal and Palek, 1992). Human and murine late

erythroprogenitor studies have been performed in Friend virus transformed cells (Koury et al, 1987 and Hanspal et al, 1992a). These cells are sensitive to erythropoietin and can be induced into terminal stages of erythropoiesis. Despite major differences between avian and mammalian erythrocytes, marked similarities in the biosynthesis of their membrane protein skeletons have been observed.

In the early stages of MEL cell differentiation (reviewed by Hanspal and Palek, 1992), α and β spectrin, ankyrin, band 4.1 protein and actin are constitutively synthesised. There is a 2-3 fold excess synthesis of α over β spectrin (Lenhert and Lodish, 1988). Band 3 mRNA and protein synthesis at this stage is low. A stable membrane skeleton does not form and the excess protein is rapidly degraded. α Spectrin is degraded via the lysosomal system and has been shown to be ubiquitinated in the αII and αV domains (Corsi et al, 1997).

Chemical induction of cultured cells results in increased haemoglobin production, a 1.5-2 fold increase in the synthesis of the constitutive membrane skeleton components and a decrease in the catabolism of these proteins. Concomitant with this is a 10-50 times upregulation of band 3 mRNA and protein production (Hanspal et al, 1992b) with co-translational insertion of band 3 into the membrane.

The final maturation of Friend virus transformed cells follows a similar pattern to that seen in MEL cells. Erythropoietin induced terminal maturation (Koury et al, 1987 and Hanspal et al, 1992b) causes decreased spectrin subunit and ankyrin synthesis but increased recruitment of these proteins into the membrane with an increase in bands 3

and 4.1 synthesis. Protein 4.1 is the last component to be assembled into the skeleton despite its presence in the membrane early on in maturation. Thus protein 4.1 interaction with skeleton components is upregulated; the control of this is unknown but may be a function of differential stage specific mRNA splicing (Conboy et al, 1991). Proteins assembled into the skeleton show protection from catabolism as evidenced by their increased half lives. The skeleton starts to be stabilised on the membrane at the time of the appearance of the band 3 protein. This would appear to be the result of attachment of spectrin via the high affinity interaction with ankyrin to band 3. However normal levels of erythrocyte membrane skeletal components are assembled on the membrane during the differentiation of red cells lacking band 3 (Peters et al, 1996). Thus the full mechanism of cytoskeleton assembly is not fully understood.

The assembly of the erythrocyte membrane protein skeleton therefore demonstrates the principles of ultrastructural self-assembly via temporal and spatial controls. The temporal induction of component synthesis and down-regulation of catabolism controls the availability of the membrane protein skeletal components. The co-translational insertion of band 3 into the membrane is a spatial control aided by the regulation of protein 4.1 interactions timed with terminal maturation. These lead to the self-assembly of the ultrastructure on the unstable pre-existing skeleton template.

Morrow and Marchesi (1981) have proposed a model to explain why the modulation of the interactions between components is not a persistent feature of membrane protein

skeleton biosynthesis. In their model, the cytoplasmic concentrations of skeleton proteins are small and the association affinities low. Therefore inappropriate cytoplasmic self-assembly does not occur spontaneously to any great extent. It is the super-concentration of protein components within the minimal volume of a "narrow submembraneous shell" (Morrow and Marchesi, 1981) by high affinity attachments to the integral proteins, that forces the equilibria to favour self-assembly. However the band 3 protein is not an absolute requirement for stable membrane protein skeleton assembly (Peters et al, 1996) and the mechanisms for self-assembly are thus more complicated than suggested by this simple model.

1.5 Hereditary haemolytic anaemia

1.5.1 Forces acting on the erythrocyte membrane in the circulation

The forces on the erythrocyte lipid bilayer during its lifetime in circulation can be divided into those acting horizontally, in the plane of the membrane, and those acting vertically at right angles to the membrane's plane (Palek, 1986). These forces are withstood by different components of the two-dimensional array of the cortical membrane protein skeleton that supports the erythrocyte lipid bilayer. A weakened membrane skeleton results in permanent erythrocyte deformation and a range of effects from a non-pathological altered erythrocyte morphology to severe haemolysis and anaemia.

Hereditary anaemias are classified according to the alterations in red cell morphology observed on peripheral blood smears (reviewed by Palek and Jarolim, 1995) and include hereditary elliptocytosis (HE), hereditary pyropoikilocytosis (HPP), hereditary spherocytosis (HS) and South East Asian ovalocytosis. Hereditary anaemia is differentiated from other cases of anaemia caused by underlying pathological states such as infection, by the existence of an erythrocyte membrane skeleton protein defect. The extensive investigations of the underlying mutations causing hereditary anaemia has greatly enhanced the understanding of skeleton protein functions and structures. During their passage through the spleen, erythrocytes are mechanically stressed (Chen and Weiss, 1973 and Weiss, 1983) and require a high degree of elasticity and deformability to survive. This is the normal mechanism for removing old or damaged red cells. Erythrocytes unable to withstand the rigours of the circulation due to an underlying membrane protein defect are recognised by the spleen and removed prematurely. In severe cases the rate of removal is not matched by the rate of erythropoiesis causing anaemia.

Although hereditary anaemias are classified according to alterations in red cell morphology, biophysically it is more useful to consider the effects of membrane skeleton defects with reference to the direction of forces acting on the red cell membrane. The mechanistic and morphological descriptions are related because particular protein defects resulting in susceptibilities to specific forces cause defined morphological changes. Figure 5 is a diagram of the erythrocyte membrane skeleton

with the protein skeleton components classified according to the direction of force that they resist.

Horizontal forces: Hereditary elliptocytosis (HE) and pyropoikilocytosis (HPP)

Horizontal forces are defined as those acting in the plane of the erythrocyte membrane (Palek, 1986). Detailed in figure 5 are the components of the membrane protein skeleton that withstand horizontal forces. The most important is the self-association of dimers into tetramers but also included are spectrin-junctional complex attachments. The lipid bilayer has a low intrinsic strength and horizontal forces permanently deform weakened erythrocytes into elliptocytes or in severe cases fragment cells. The severity of the deformation is related to the extent of the underlying functional defect (Coetzer et al, 1987). Mutations in α and β spectrin that influence dimer self-association (reviewed by Delaunay and Dhermy, 1993, Palek and Jarolim, 1995 and Gallagher and Forget, 1996) are the major causes of HE. This disease is characterised by elliptocytes on the peripheral blood smear (Palek and Jarolim, 1995). In many cases of HE caused by a mutant spectrin, the mutation alters the molecule's structure such that it is detected by an altered tryptic digest pattern. There is typically a decreased spectrin αI domain molecular weight seen on SDS PAGE. In addition to mutant spectrin, mutations of glycophorin C and the band 4.1 protein also cause HE.

HPP, a severe form of HE, is characterised by poikilocytes and microspherocytes on peripheral blood smears (Palek and Jarolim, 1995). The cause is a spectrin mutation that reduces spectrin dimer self-association combined with a spectrin synthesis defect

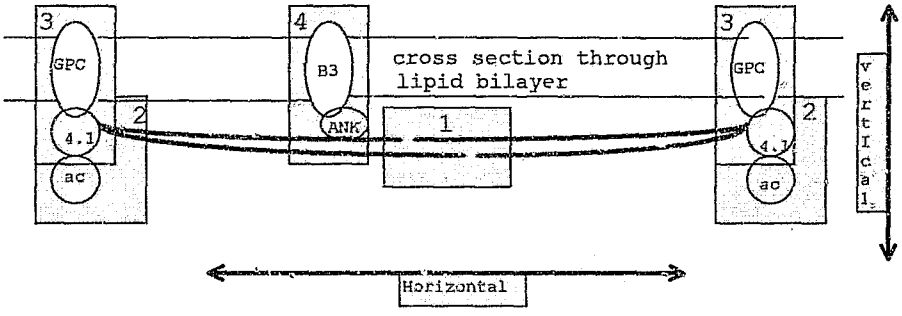


Figure 5.

Delineation of the membrane skeleton proteins that maintain horizontal and vertical interactions in the erythrocyte membrane. Protein interactions that withstand horizontal forces, in the plane of the lipid bilayer, are shown in lightly hatched boxes: (1) spectrin dimer self-association and (2) spectrin-protein 4.1-actin. Protein interactions that withstand vertical forces, acting at right angles to the plane of the erythrocyte lipid bilayer, are (3) spectrin-protein 4.1-glycophorin C (GPC) and (4) spectrin-ankyrin-band 3. Abbreviations and colour key: α spectrin = red line, β spectrin = green line, ankyrin = ANK, actin = ac and band 4.1 protein = 4.1. (Diagram adapted from Palek, 1995).

that results in spectrin deficiency in the membrane (Coetzer and Palek, 1986 and Gallagher and Forget, 1993).

Clinical features and diagnosis of hereditary elliptocytosis and hereditary pyropoikilocytosis

HE and HPP are marked by the presence of abnormally high levels of elliptocytic or poikilocytic red blood cells on peripheral blood smears (Palek and Jarolim, 1995). In severe cases the red cells show an increased osmotic fragility. HE individuals present with variable symptoms from an assymptotic carrier state to life threatening anaemia that requires splenectomy.

Crude low temperature spectrin extracts reflect the relative levels of spectrin dimers and tetramers in the membrane (Liu and Palek, 1980). HE patients with an underlying spectrin mutation typically show increased dimer levels indicating a spectrin self-association defect. In general mild HE is not associated with decreased membrane spectrin content. However in severe cases of HE and HPP spectrin content is diminished.

Common HE is inherited in an autosomal dominant fashion (Palek and Jarolim, 1995). Heterozygous individuals are commonly assymptomatic in part due to those with α spectrin defects where the excess synthesis of α spectrin during erythrocyte maturation dilutes the effect of α spectrin mutations in heterozygotes. Typically severe pathological states are found in homozygous or compound heterozygous individuals.

Southeast Asian ovalocytosis.

Southeast Asian ovalocytosis is a dominantly inherited disease found in Malaysia, Papua New Guinea and Indonesia (Palek and Jarolim, 1995) and in the Cape Coloured population of South Africa (Coetzer et al, 1996). Ovalocytic erythrocytes are identified by a transverse ridge. The cells are less deformable than normal and show decreased osmotic fragility. The underlying mutation is in band 3 (Jarolim et al, 1991, Tanner et al, 1991 and Liu et al, 1990). The high incidence of ovalocytosis in south east Asian populations is thought to be a consequence of the increased resistance of ovalocytes to invasion by malarial parasites (Hadley et al, 1983).

Vertical forces: hereditary spherocytosis (HS)

The membrane protein associations that stabilise the erythrocyte lipid bilayer against vertical forces (Palek, 1986) are shown in figure 5. These interactions attach the protein skeleton to the membrane lipid bilayer. The skeleton covers approximately half the area of lipid (Liu and Derick, 1992) and decreased stability of these interactions results in lipid loss in the circulation. This manifests in microvesiculation; microvesicles are membrane protein skeleton-free lipid vesicles that bud from abnormal erythrocytes. The erythrocyte loses lipid surface area after budding and has a spherical morphology (Palek and Jarolim, 1995).

Clinical features and diagnosis of hereditary spherocytosis

The HS phenotype is denoted by spherocytic red blood cells that lack a central concavity (Palek and Jarolim, 1995). The cells are weakened and show increased

osmotic fragility. The severity of the disease is highly variable from life threatening to asymptomatic status. Clinically the severe anaemia is improved by splenectomy.

The underlying molecular causes of HS are more varied than for HE (Palek and Jarolim, 1995). Membrane spectrin content is usually reduced but spectrin extracted from HS patients typically shows normal spectrin tetramer levels. Mutations in α and β spectrin, ankyrin, band 3 and protein 4.2 have been described for HS.

1.6 Aim

The aim of this project was to identify the molecular basis of the spectrin defect underlying an atypical case of recessively inherited HE in two probands in a white South African kindred. In addition, the utility of a competitive spectrin-ankyrin binding assays for screening multiple spectrin samples for binding defects was evaluated.

1.7 Strategy for experimental work

The probands' spectrin showed reduced dimer self-association attributable to either monomer, an ankyrin binding defect which is an altered β spectrin function and altered αII domain structure residing on α spectrin. These facts were evidence that an apparent defect in one subunit, such as the lowered ankyrin binding, was the manifestation of a primary defect of the other chain. Thus the aim of the protein analysis was to identify the subunit primarily responsible for each defect. This was

achieved by the separation and reassociation of control and proband spectrin monomers into hybrid spectrins (hSp). hSp were then assayed function and structure. Guided by the results of the protein data, the second part of the experimental work was an analysis of the relevant mRNA and genomic DNA regions in the probands.

2. Protein methods

2.1 Materials

All reagents were of analytical or molecular biology grade and solutions were prepared in MilliQ water (Millipore, USA).

2.2 Subjects

2.2.1 Clinical features of probands 1 and 2 (P1 and P2)

Probands 1 (P1) and 2 (P2) are the same individuals identified in the literature as AD and CG respectively (Gomperts et al, 1973, Coetzer and Zail, 1981, Coetzer and Zail, 1982 and Zail and Coetzer, 1984). P1 and P2 are white females aged 40 and 45 respectively with well compensated haemolytic anaemia; both probands were splenectomised in childhood. P1 and P2 had reticulocyte counts of 1-1.5% and 4-7% respectively. Peripheral blood smears from P1 and P2 show predominantly elliptocytes with some poikilocytes and budding. Erythrocyte osmotic fragility is also increased.

Biochemical features of P1 and P2 spectrin

Both probands had a 30% reduction in membrane spectrin content over normal values (Coetzer and Zail, 1984). This finding is unusual for HE since spectrin deficiency is more commonly associated with HPP (Coetzer and Palek, 1986). Crude 4°C extracts of P2 spectrin had 20% and 22% dimers, expressed as percentages of the spectrin dimers and tetramers, compared to an average control level of 7.5% dimers (Coetzer

and Zail, 1982). These levels are not high enough to explain the severity of the clinical symptoms. 37°C crude extracts showed normal levels of dimer. Dimer-tetramer conversion assays of P2 spectrin confirmed the existence of a spectrin dimer self-association defect. P1 and P2 spectrin dimer-ankyrin binding assays indicated a severe defect resulting in an approximately 50% reduction in ankyrin binding (Zail and Coetzer, 1984). Initial analyses of tryptic digests of P2 spectrin suggested normal structure (Coetzer and Zail, 1981). However subsequent analysis identified an alteration of the αII domain in P1 and P2 spectrin (S. Lambert, private communication). This structural alteration was further investigated in this work.

Biochemical features of the parents of the probands

The parents of the probands are both haematologically normal. Biochemically the father, now deceased, showed a spectrin-ankyrin binding defect (Zail and Coetzer, 1984) with approximately 25% of the control binding level. The mother's spectrin was found to be normal.

HS patient DB

Patient DB showed spherocytes on peripheral blood smears. A spectrin/band 3 ratio of 0.94 was measured in membrane studies with control values of 1.07+/- 0.07 for the laboratory. Thus DB showed a mild spectrin deficiency implying a possible spectrin-ankyrin binding defect.

2.3 Control blood samples

Control blood samples were drawn from healthy adult laboratory staff volunteers.

2.4 Ethical clearance

Ethical clearance was granted by the University of the Witwatersrand Medical School Ethics Committee in clearance certificate no. 25/1/92.

2.5 Hybrid spectrin (hSp) preparation

hSp were prepared by the reassociation and renaturation of control and patient α and β spectrin monomers. Purified spectrin dimers were urea denatured (Yoshino and Marchesi, 1984) and the monomers separated by low pressure ion exchange chromatography (LeComte et al, 1990b). Pure β spectrin and α spectrin fractions contaminated with β spectrin were eluted from the ion exchange column on a NaCl gradient. Control and proband monomers were reassociated and renatured by dialysis against isotonic solution to produce hSp. To minimise proteolysis all procedures were undertaken at 8°C with solutions cooled on ice.

2.5.1 Spectrin extraction

Up to 10mg of crude spectrin extracts containing low levels of the bands 4.1 and 5 proteins were prepared from red blood cells. Washed erythrocytes were lysed in hypotonic solution (Dodge et al, 1963), haemoglobin free red cell membrane ghosts were prepared (Coetzer and Palek, 1986) by repeated after which spectrin was extracted.

Lysis buffer

3mM $NaPO_4$, pH 8.0

0.1mM Ethylenedinitrilo tetra-acetic (EDTA)

0.1mM Phyenyl-methyl-sulfonyl fluoride (PMSF)

Disodium phosphate and EDTA were dissolved in 950ml of water and adjusted to pH 8 with 0.2% sodium dihydrogen phosphate. 400mM PMSF (Boehringer Mannheim, Germany) dissolved in dimethyl sulfoxide (DMSO) was added slowly in a dropwise fashion to a final concentration of 0.1mM while the solution was rapidly stirred. The buffer was made up to 1 litre and cooled on ice. Fresh buffer was made for membrane preparations.

Extraction buffer

0.1mM $NaPO_4$, pH 8.0,

0.1mM EDTA,

0.1mM DTT

60ml blood samples were collected in acid citrate dextrose tubes (Vacutainer, USA), stored on ice and processed within 4 hours of collection. Samples were centrifuged at 800g for 10 minutes at 4°C to separate plasma, red cell and buffy coat fractions. Plasma fractions were aspirated and discarded, buffy coats were stored at -70°C and red cells were transferred to precooled 15ml polypropylene tubes on ice. Red cells were washed 4 times in 12ml ice cold 0.9% saline and pelleted between washes by centrifugation at 1,700g for

10 minutes at 4°C. 5ml aliquots of cells were transferred to prechilled 45ml centrifuge tubes (Beckman, USA) and lysed with 30ml of ice cold lysis buffer. Samples were pelleted by centrifugation in a prechilled JA17 rotor (Beckman, USA) at 22,000g for 15 minutes at 4°C. Supernatants were aspirated and the loose red cell membrane pellets resuspended. Dense white cell "buttons" visible on the sides of tubes after resuspension of the red cell membranes were aspirated. Three to four lysis buffer washes cleared haemoglobin from control membranes leaving a white cloudy ghost pellet. Ghosts from HE and HS individuals retained a pink colour due to the persistent presence of haemoglobin. 100mM Pefabloc protease inhibitor (Boehringer Mannheim, Germany) was added to ghosts to a final concentration of 100μM and ghosts were then incubated on ice for 10 minutes. Pefabloc treatment was omitted if ghosts were to be used for spectrin extractions where the crude extract was for immediate tryptic digestion.

Extraction of spectrin dimers and tetramers

Pelleted red cell ghosts were resuspended in 30ml fresh ice cold extraction buffer and pelleted by centrifugation at 30,000g for 20 minutes at 4°C. Supernatants were aspirated to a volume approximately half that of the pellet volume. Pellets were resuspended and spectrin dimers extracted by incubation of the ghosts at 37°C for 30 minutes. Tetrameric spectrin was extracted by incubation of the ghosts for 20 hours on ice. Membranes and spectrin extracts were separated by centrifugation in a Ti50 rotor (Beckman, USA) at 130,000g for 30 minutes at 4°C. Supernatants, which contained crude spectrin extracts,

were aspirated, pooled and stored on ice. Protein yields were quantitated using the BioRad Protein Determination Kit (section 2.8.1).

2.5.2 Purification of spectrin dimers by size exclusion chromatography

Column buffer

20mM Tris-HCl, pH 8.0,

1mM EDTA,

10mM ß-mercaptoethanol.

Column buffer was filtered through a 22µm filter (MSI, USA) and cooled to 4°C. This step allows size exclusion chromatography column purified spectrin dimers to be applied to an ion exchange HPLC column without requiring further filtration.

Spectrin dimers were purified using chilled buffers to avoid conversion to tetramers. 10-13mg of freshly prepared crude control and proband spectrin dimer extracts, concentrations between 1.5 and 2.5mg/ml, were loaded onto a Sepharose CL4B column (90cm X 3cm) (Pharmacia, Sweden) equilibrated in fresh column buffer. Fractions were eluted in column buffer and the absorbance of column eluate was monitored at 280nm. high molecular weight complexes eluted in the void volume in the first peak, spectrin tetramers eluted later in the first peak. Spectrin dimers eluted in the second and much largre third peak. Spectrin dimers were eluted in column buffer at a flow rate of approximately 20ml/hour into 4ml fractions which were pooled and stored on ice.

2.5.3 Ion exchange chromatographic separation of spectrin monomers

Spectrin denaturation and ion exchange column loading

Solutions

X 2 Urea denaturation solution

6M urea,

20mM Tris-HCl, pH8.0,

10mM ß-mercaptoethanol,

1mM EDTA,

0.3M NaCl.

Ion exchange buffer A

3M urea,

20mM Tris-HCl, pH8.0,

10mM ß-mercaptoethanol,

1mM EDTA,

0.25M NaCl.

Ion exchange buffer B

3M urea,

20mM Tris-HCl, pH8.0,

10mM ß-mercaptoethanol,

1mM EDTA,

0.40M NaCl.

Isotonic KCl buffer

10 mM $NaPO_4$, pH 7.5

0.13M KCl

0.02M NaCl

1mM EDTA

1mM DTT or 10mM β-mercaptoethanol

All solutions were filtered through 22μm filters (MSI, USA) and cooled on ice.

5mg samples of size exclusion column purified spectrin dimers at concentrations between 0.1-0.2mg/ml were denatured in autoclaved Erlenmeyer flasks for 1 hour on ice by the addition of equal volumes of X 2 urea denaturation solution prior to the separation of the spectrin monomers by ion exchange chromatography.

NaCl solutions for ion exchange chromatography were dissolved in a common column buffer comprising: 3M urea, 20mM Tris-HCl, pH8.0, 1mM EDTA, 10mM β-mercaptoethanol. The separation procedures were performed at 8°C to reduce proteolysis. An HR 5/5 MonoQ ion exchange chromatography column (Pharmacia, Sweden) was used as instructed by the manufacturer. Flow rates were kept constant at 1ml/min.

Denatured spectrins were loaded onto a Mono-Q HR 5/5 ion exchange chromatography column (Pharmacia, Sweden) equilibrated in 150mM NaCl/column

buffer. 5ml buffer A were then passed through the column prior to the elution of spectrin monomers.

β Spectrin elution

β Spectrin was eluted on a 10 minute gradient from 150mM-350mM NaCl in column buffer. Pure β spectrin eluted from 280mM NaCl and 2ml fractions were collected and pooled. 30ml of 350mM NaCl solution were then pumped through the column at 1ml/min to remove residual β spectrin.

α Spectrin elution

α Spectrin was eluted on a 350mM-400mM NaCl gradient (initial 20% buffer B/80% buffer A to 100% buffer B) over 30 minutes and collected in 0.5ml fractions. 100mM Pefabloc (Boehringer Mannheim, Germany) was added to a final concentration of 100μm to the fractions which were stored on ice. Storage tubes were firmly capped to stop evaporation.

Concentration of pooled β spectrin fractions

Pooled β spectrin samples volumes, typically 10-12ml, were reduced to 2-3ml at 8°C. 3ml Slide-A-Lyser (Pierce, USA) dialysis cassettes were filled with dilute β spectrin. The Slide-A-Lyser dialysis membrane windows were covered in PEG 20,000 (Fluka, Switzerland) and sample volumes were reduced to approximately 0.5 ml. The dialysis cassettes were then refilled and the volumes reduced as before. This process was repeated until the volume of each β spectrin fraction was approximately 2 ml.

Concentrated β spectrins were transferred to microfuge tubes and stored on ice. The A_{280} values varied between 0.3-0.7 which was a 10-20 times increase over the initial concentration. β Spectrin monomers adsorbed to dialysis membranes causing significant yield loss. Therefore dialysis cassette membrane surfaces were kept moist until the A_{280} values of samples were satisfactory. In the event that samples required further concentration, the same dialysis cassettes were used.

Reassociation of spectrin monomers into hSp dimers

Quantitation of the β spectrin contamination of α spectrin fractions

Aliquots of α spectrin fractions were separated by SDS PAGE for the purpose of determining by densitometric gel scanning the β spectrin content relative to the amount of α spectrin.

10ul aliquots of the initial 20 fractions collected from an α spectrin peak were electrophoresed on 8cm X 7cm 8% discontinuous SDS PAGE minigels. Discontinuous SDS PAGE gels (Laemmli, 1970) were poured using the components given in table 1. Electrophoresis in Laemmli electrophoresis buffer, table 1, was performed at 100 volts for 2.5 hours at 10°C in a Mighty Small minigel apparatus (Hoefer, USA). Gels were stained with Fast Stain (BDH, UK) for 2 hours and extensively destained for 10-12 hours with frequent changes of water. The α and β spectrin content of fractions was measured by densitometric scanning of gel lanes (section 2.8.4). The relative amounts of each monomer were expressed as a percentage of the total protein (α+β). α Fractions with a β spectrin contamination

	Resolving gel	Stacking gel
30% Acrylamide (w/v)	6.0ml	500µl
1% Bisacrylamide (w/v)	1.6ml	390µl
4 X Gel Buffer	5.7ml*	750µl**
Water	9.0ml	1.3ml
20% SDS	113µl	15µl
TEMED	12.5µl	300µl
10% APS	100µl	60µl

Table 1

Components of 8% Laemmli discontinuous SDS PAGE gels used for analysing α and β spectrin monomer levels in ion exchange chromatography fractions.

*X4 resolving gel buffer: 1.5M Tris-HCl pH 8.3

**X4 stacking gel buffer: 0.5M Tris-HCl pH 6.8

Laemmli electrophoresis buffer:

0.025M Tris pH8.8

0.192M Glycine

0.1% SDS

below 20% were pooled and the A_{280} value measured. The percentage α and β spectrin in each pooled α spectrin fraction was measured from the average of two densitometric quantitations of SDS PAGE gel lanes containing 2.5 and 5 absorption units (A_{280} X μl) of each pooled sample.

Derivation of the calculation for the amount of β spectrin added to pooled α spectrin fractions for reassociation of hSp

hSp were reassociated with an estimated 1α:1β molar ratio of spectrin monomers that included the contaminating β spectrin in pooled α spectrin fractions. The β spectrin added to a pooled α spectrin sample was based upon the estimated amount of α spectrin free to bind added β monomer. The calculation of the amounts of pooled α and β spectrin samples mixed to reassociate into hSp is derived below. In a pooled α spectrin sample, the total amount of spectrin in absorbance units, $[AU]_T$, is given by the product of the A_{280} value (α_{280}) and the volume (v).

$$[AU]_T = \alpha_{280} \text{ X } v$$

The amount of α spectrin, $[AU]_\alpha$, and β spectrin, $[AU]_\beta$, in the sample are then given by the products of the relative percentages of each monomer, %α and %β respectively, measured from densitometric gel scans and the total amount of spectrin.

$$[AU]_\alpha = \%\alpha \text{ X } [AU]_T/100$$

$[AU]_{\beta} = \%\beta \text{ X } [AU]_T/100$

The β spectrin contamination in an α spectrin sample reduces the available α spectrin free, $[AU]_{\alpha free}$, to bind added β monomer. $[AU]_{\alpha free}$, is the difference between the amounts of β and α spectrin.

$$[AU]_{\alpha \text{ free}} = [AU]_{\alpha} - [AU]_{\beta}$$

The amount of β spectrin, $[AU]_{\beta \text{ added}}$, added to a pooled α spectrin sample was equal to $[AU]_{\alpha \text{ free}}$ multiplied by a correction factor of 1.1 for the differences in molecular masses (Winkelmann et al, 1990 and Sahr et al, 1990). It was assumed that the extinction coefficients of urea denatured α and β spectrin at 280nm are the same.

$$[AU]_{\beta \text{ added}} = 1.1 \text{ X } [AU]_{\alpha \text{ free}}$$

Experimentally β spectrin yields were limiting and to form the four possible hSp from a preparation of control and proband monomers, each β spectrin fraction was halved and pooled α spectrin added.

hSp reassociation and renaturation

Spectrin monomers were mixed and dialysed in 3ml Slide-A-Lyser dialysis cassettes (Pierce, USA) at 4°C. Dialysis was for 24 hours against 4 changes of isotonic KCl buffer (section 2.5.3) each one at least 200 times the volume of dialysed sample. The KCl buffer contained β-mercaptoethanol for the first three changes and DTT in the

last. The amounts of hSp were quantitated using a commercially available protein determination kit (section 2.8.1).

2.6 *Spectrin structural analysis: tryptic peptide mapping*

Spectrin tryptic digest peptides were mapped by a two dimensional separation incorporating isoelectric focusing (IEF) and SDS PAGE using a modified version of the method of O'Farrell (1975).

2.6.1 Mild limited isotonic tryptic digestion

X 10 isotonic digestion buffer

100mM $NaPO_4$,pH 7.5

1.5M NaCl

50mM EDTA

50mM ß-mercaptoethanol

Hypotonic crude spectrin extracts were made isotonic with X10 digestion buffer. hSp were digested in isotonic KCl buffer (section 2.5.3). Spectrin samples were digested with TPCK Trypsin (Worthington, USA) for 18 hours on ice using an enzyme to substrate ratio of 1:100 (w/w) (Speicher et al, 1980). Digestions were stopped by adding 50mM PMSF (Boehringer Mannheim, Germany) in DMSO to a final concentration of 5mM and then boiling samples for 2 minutes. 200 µg aliquots of digests were freeze dried and stored at -20°C.

2.6.2 First dimension separation: isoelectric focusing (IEF) gel preparation

Solutions

Solutions are designated letters according to O'Farrell (1975).

A)Lysis buffer: 9.5M urea, 2% w/v NP-40, 1.6% pH 5-7 Biolyte (Biorad, USA), 0.4% 3-10 Biolyte (Biorad, USA,), 5% ß-mercaptoethanol.

D) Acrylamide stock: w/v 28.38% acrylamide and 1.62% bis-acrylamide.

E) 10% (w/v) Nonidet P40 (Sigma, USA) in water.

H) Gel overlay solution: 8M urea.

I) Anode electrode solution: 0.01M H_3PO_4.

J) Cathode electrode solution: 0.02M NaOH, degassed by stirring under vacuum for 1 hour.

K) Sample overlay solution: 9M urea, 0.8% Biolyte 5-7 (Biorad, USA), 0.2% Biolyte 3-10 (Biorad, USA).

O) SDS sample buffer: 0.063M Tris-HCl pH 6.8, 10% w/v glycerol, 5% v/v ß-mercaptoethanol, w/v 2.3% SDS, 1% Bromophenol blue.

Solutions A, F and K were made up as stocks and stored at -20°C. Solution D was freshly prepared for each batch of gels.

10 glass tubes (130 X 2.5mm) were sealed with a triple layer of laboratory film and held vertically in a rack. 5.5g of urea, 1.33ml D, 3.77ml MilliQ water (Millipore,

USA), 0.4ml Biolyte 4-7 and 0.1ml Biolyte 3-10 (Biorad, USA) were mixed in a 20ml side arm test tube and warmed to 37°C to dissolve the urea. The solution was degassed by gentle agitation under vacuum for 1 minute. 200µl of NP-40 (Sigma, USA), 10µl of fresh 10% APS and 7µl of TEMED were added and mixed without foaming the solution. Gel mixture was syringed into each tube to a height of 10cm and overlaid with 20µl H. Gels were polymerised at room temperature for 2 hours. Solution H overlays were then aspirated and replaced with 20µl of A for a further 2 hours. Gels could be stored at room temperature for 3 days after sealing tubes with laboratory film.

IEF of protein samples

IEF gels were overlaid with fresh solution A, placed into an electrophoresis tank and the bottom reservoir filled with solution I. Tubes were filled with solution J which was layered over solution A. The upper buffer reservoir of the electrophoresis tank was then filled with solution J and gels prefocused at 200 volts for 15 minutes, 300 volts for 30 minutes and 400 volts for 30 minutes. The top buffer reservoir was then emptied and the liquid inside each tube aspirated with a glass pipette. 200µg samples of spectrin tryptic digests dissolved in 50µl of A were loaded onto gels, overlaid with 10µl K and the tubes refilled with J. The upper reservoir was filled with fresh J and the samples focused at 400 volts for 12 hours followed by 1 hour at 800v. Gels were extruded into 15ml tubes and equilibrated for 2 hours in 10ml solution O. Gels could be stored at -20°C for several months.

Measurement of IEF gel pH gradients

A focused blank IEF gel was extruded and sliced into 1cm lengths. Slices were placed into 1ml of MilliQ water (Millipore, USA) and shaken for 1 hour. The pH of each 1ml solution was measured. From this a curve of pH versus distance along the IEF gel was plotted.

2.6.3 Second dimension: Discontinuous Laemmli SDS PAGE

10% Laemmli (1970) discontinuous gels were poured using the components listed in table 2. Stacking gels were polymerised without combs and IEF gels were laid flat onto gel surfaces and held in place by a 1% agarose solution dissolved in IEF solution O. A well was formed in the agarose to one side of each IEF gel for electrophoresing solubilised erythrocyte membrane protein molecular weight markers.

Gels were electrophoresed overnight at 75 volts and stopped as the dye front from the molecular weight markers was about to elute from the gel. Gels were stained in Coomassie blue and destained (section 2.8.3).

2.7 Spectrin functional analyses

2.7.1 hSp dimer to tetramer conversion assays

The concentrations of aliquots of hSp were determined using the BioRad protein determination kit (section 2.8.1) and equalised to the value of the lowest by dilution with ice cold isotonic KCl buffer (section 2.5.3). 100mM Pefabloc (Boehringer

	Resolving Gel	Stacking gel
40% Acrylamide/1.5% Bisacrylamide	15ml	665µl
X 4 Gel Buffer	15ml*	2.5ml**
Water	23.1ml	4.6ml
20% SDS	300µl	50µl
0.2M EDTA	600µl	100µl
0.5% TEMED	3ml	1ml
1.5% APS	3ml	1ml

Table 2.

Components for 10% discontinuous Laemmli SDS PAGE gels for the second dimension separation of spectrin tryptic digest pepitdes.

*X4 resolving gel buffer: 1.5M Tris-HCl pH 8.3

**X4 stacking gel buffer: 0.5M Tris-HCl pH 6.8

Laemmli electrophoresis buffer:

0.025M Tris pH8.8

0.192M Glycine

0.1% SDS

Mannheim, Germany) was added to each to a final concentration of 100μM. 10μg hSp aliquots were kept on ice for measurements of the initial dimer and tetramer levels. Triplicate 10μg samples of isotonic hSp were incubated in a water bath at 30°C for 6 hours. After incubation, samples were analysed by non-denaturing gel electrophoresis (Liu and Palek, 1980).

Non-denaturing gel preparation

The oligomeric states of spectrins were analysed on non-denaturing acrylamide/agarose gels (Liu and Palek, 1980). Glass tubes (12cm X 3mm) were sealed with a triple wrapping of laboratory film and a mark was made on each 8 cm from the bottom. Tubes were submerged in a 37°C water bath to a height of 9cm. The gel reagents shown in table 3, except for the APS and TEMED, were mixed in an Erlenmeyer flask and could be kept at 42°C for several hours. Gel mixture was poured into a 100ml beaker on a magnetic stirrer, APS and TEMED were added, the solution was stirred for 10 seconds and then transferred to a 50ml syringe barrel to which a 5cm length of tubing (Tygon, USA) was attached. Gel mixture was poured into the glass tubes and the gel mixture then aspirated from each to the 8cm mark with a Pasteur pipette. Water was gently layered over each gel using a syringe and 23 gauge needle. Tubes were transferred to an ice bath for 3 minutes to set the agarose followed by polymerisation at room temperature for 30 minutes. A sharp interface between the agarose cap and the agarose-acrylamide at the tops of gels indicated polymerisation was complete. The water overlay was replaced with X 1 Fairbanks buffer (table 3), the

Solutions	ml
40% acrylamide/ 1.5% bisacrylamide	3.1
1% agarose	15
X 10 buffer	5
water	22
1.5% APS	2.5
0.5% TEMED	2.5

Table 3.

Components for non-denaturing agarose/acrylamide gels for the analysis of the oligomeric state of spectrin samples.

X 10 Fairbanks buffer:

400mM Tris-acetate, pH 7.4,

200mM sodium acetate,

20mM EDTA.

Electrophoresis buffer: X 1 Fairbanks buffer with 80 µl β-mercaptoethanol.

glass tubes were sealed with Parafilm (American National Can, USA) and could be stored for several months at 4°C.

Analysis of the oligomeric state of spectrin

Spectrin oligomeric analyses were undertaken at 4°C with chilled buffers. An electrophoresis tank was assembled with gels and both buffer reservoirs filled with ice cold X1 electrophoresis buffer (table 3). Ice cold 2M sucrose, 1% bromophenol blue was added to 10-15µg spectrin samples to a final concentration of 200mM sucrose, 0.1% bromophenol blue. Samples were loaded onto gels with a microsyringe (Hamilton, Switzerland). Electrophoresis was for 16 hours at 20 volts and 4°C. Gels were extruded into 15ml tubes, stained with Coomassie blue and destained (section 2.8.3). Spectrin oligomers were quantitated by densitometric scans of destained gels (section 2.8.4).

2.7.2 Spectrin-ankyrin binding assays

^{125}I radiolabelling of spectrins

Spectrin dimers (section 2.5.2) and hSp were radiolabelled with Bolton and Hunter reagent (Zail and Coetzer, 1984). 65µCi Bolton and Hunter reagent (Amersham, UK)/mg spectrin were pipetted into 5ml glass test tubes at room temperature and the tubes corked. Corks were pierced with two 20 gauge needles: one attached to a nitrogen gas supply (Afrox, South Africa) and the other to a charcoal trap (Amersham, UK). The benzene in the reagent was dried under a gentle nitrogen stream for 5-10

minutes and test tubes were placed on ice. The nitrogen stream was continued for a further 5 minutes.

500mM sodium borate, pH 8.0, was added to a final concentration of 50mM to spectrin samples. Samples were then added to the dried Bolton and Hunter reagent on ice. Reactions were incubated for 1 hour and stopped by the addition of 100mM glycine to a final concentration of 10mM (Bolton and Hunter, 1973).

Unincorporated label was separated from labelled spectrin on 10cm X 1cm Sephadex G-50 columns (Pharmacia, Sweden) pre-equilibrated in isotonic KCl buffer (section 2.5.3). The precaution of working in a fume hood behind radioactivity safety screens required that columns be run at room temperature. To reduce the risk of proteolysis and oligomerisation of spectrin samples, columns were equilibrated with ice cold isotonic KCl buffer and stored at 4°C until required. Spectrin eluted in the column void volumes within 2-3 minutes and 0.5ml fractions, collected manually, were stored on ice. Labelled spectrin fractions were identified using a hand held Gamma radiation monitor and 10µl of each candidate fraction was dispensed into 0.5ml dilute Bradford protein dye (section 2.8.1). Fractions with a qualitatively strong blue colour were pooled. ^{125}I radiolabelled spectrins were dialysed in Slide-A-Lysers dialysis cassettes (Pierce, USA) against isotonic KCl buffer at 4°C. Dialysis continued until dialysate radioactivity was less than 100cpm/ml typically requiring 4-5 buffer changes of a X500 volume over 36 hours. Labelled spectrins were quantitated using the BioRad protein determination kit (section 2.8.1).

Measurement of unincorporated ^{125}I label

The percentage free ^{125}I Bolton and Hunter reagent in labelled samples was measured by acid precipitation of spectrin samples (Ausubel et al.). The total initial radioactivity of duplicate 20µl labelled spectrin samples dispensed into 0.5ml microfuge tubes was measured in a Hydragamma (Innotron, UK). 125µl 0.1mg/ml bovine serum albumin (BSA) carrier protein and 125µl 20% trichloroacetic acid were added to each sample, proteins were precipitated on ice for 1 hour and pelleted by centrifugation at 10,000g for 30 minutes. Supernatants were pipetted into fresh tubes and residual moisture around pellets dried with cotton wool swabs. Supernatant and pellet radioactivities were measured in a Hydragamma gamma radiation counter (Innotron, UK) and expressed as percentages of the initial total radioactivity. Typically free ^{125}I Bolton and Hunter reagent radioactivity counts were less than 1-2% of the total and spectrin radioactivity was approximately 20,000cpm/µg.

Spectrin depleted inside-out vesicle (IOV) preparation

Red blood cell ghosts from an initial 6ml of blood (section 2.5.1) were treated with 100mM Pefabloc (Boehringer Mannheim, Germany) added to a final concentration of 10mM. Ghosts were portioned into two 45ml centrifuge tubes (Beckman, USA) and each aliquot resuspended in 30ml of ice cold 0.25mM EDTA, pH 8.0 (Zail and Coetzer, 1984). Ghosts were repelleted by centrifugation at 30,000g for 30 minutes at 4°C. Supernatants were aspirated and pellets resuspended in 30ml 0.25mM EDTA, pH 8.0, warmed to 37°C. Spectrin was extracted for 30 minutes by incubation at 37°C.

IOV were separated from spectrin by centrifugation at 30,000g for 30 minutes at 4°C. Supernatants were aspirated and pellets resuspended in ice cold isotonic KCl buffer (section 2.5.3) to a concentration of approximately 1mg/ml. Spectrin depleted IOV were treated again with 10mM Pefabloc and IOV protein concentration was determined (section 2.8.1). Spectrin depleted IOV could be stored on ice for up to 24 hours.

^{125}I labelled hSp-ankyrin binding assays

A range from 0-17μg of ^{125}I labelled hSp were dispensed into 0.5ml microfuge tubes on ice and volumes made up to 200μl with isotonic KCl buffer (section 2.5.3). Duplicate assays and a heat denatured blank were performed for each point. Spectrins were heat denatured at 70°C for 10 minutes for the blank assays. Assays were started by the addition of 16μg of spectrin depleted IOV in 50μl (0.32mg/ml) administered with a repeater pipette. Assays were incubated on ice for 90 minutes and stopped by centrifugation at 45,000g for 30 minutes at 4°C in a J 20.1 rotor (Beckman, USA). Supernatants were aspirated. The radioactivity of IOV pellets and ^{125}I labelled spectrin standards were measured in an Hydragamma 16 (Innotron, UK) gamma radiation counter. The measured pellet-bound radiolabelled spectrin was used to calculate the free spectrin (μg/ml) in each assay which was plotted against the amount (μg) of bound spectrin per milligram of IOV protein.

hSp-ankyrin competitive binding assays

Spectrin-ankyrin competitive binding assays (Zail and Coetzer, 1984) measured the relative capacities of hSp to inhibit control radiolabelled spectrin dimers from binding to spectrin depleted IOV.

Concentration of control spectrin dimers. The concentration of a 1mg aliquot of size exclusion chromatography purified control spectrin dimers (section 2.5.2) was increased to 1mg/ml using the polyethylene glycol method described for β spectrin (section 2.5.3). Spectrin dimers were concentrated and then dialysed overnight against isotonic KCl buffer (section 2.5.3) at 4°C. A 0.5 mg aliquot was then radiolabelled.

Binding assays. Spectrin depleted IOV, radiolabelled ^{125}I spectrin dimers and hSp samples were diluted in ice cold isotonic KCl buffer (section 2.5.3): 135μg of each hSp was diluted to 0.1mg/ml, control labelled spectrin dimers were diluted to 0.25mg/ml and the spectrin depleted IOV were diluted to 0.32mg/ml. 450μl of each hSp was heat denatured at 70°C for 10 minutes.

1-20μg of hSp and 5μl of ^{125}I labelled control spectrin dimers were aliquoted into 0.5ml assay tubes and the volumes made up to 200μl with KCl buffer. Assays were performed in duplicate with a heat denatured blank to account for non-specific binding. Included was an inhibitor free assay to measure the binding of the control spectrin dimer. Assays were started by the addition of 50μl of spectrin depleted IOV using a repeater pipette. Tubes were capped and incubated on ice for 90 minutes. Assays were stopped by centrifugation in a J20.1 rotor (Beckman, USA) at 4°C for 30

minutes at 45,000g to pellet spectrin depleted IOV. Supernatants were aspirated from each tube and residual moisture around pellets was removed with cotton wool swabs. The radioactivities of pellets and 1μg labelled spectrin dimer standards were measured in a Hydragamma 16 (Innotron, UK).

In competitive binding assays, hSp competed with radiolabelled control spectrin for ankyrin binding sites on spectrin depleted IOV. The level of labelled control spectrin dimer bound was plotted against the amount of hSp added.

Analyses of hSp used in ankyrin binding assays

Non-denaturing gel analysis of the oligomeric state of hSp. The oligomeric states of hSp used for the ankyrin binding assays were analysed by non denat ing gel electrophoresis. 10μg hSp aliquots sampled at the time of the binding assays were stored on ice and analysed as described (section 2.7.1).

Measurement of the α/β spectrin ratios of spectrin depleted IOV bound hSp. The α:β Spectrin ratios of hSp bound to IOV were measured by densitometric scanning of SDS PAGE gel separations of the solubilised proteins in ankyrin binding assay IOV pellets. After recording radioactivity measurements, ankyrin binding assay IOV pellets from binding assays in which 17.5μg of hSp were added were solubilised and the proteins separated by 3.5-17% continuous exponential gradient SDS PAGE (Fairbanks et al 1971). To measure a control α:β spectrin ratio, a control unlabelled spectrin dimer

binding assay was performed and the solubilised pellet protein separated on SDS PAGE gels with the other binding assay samples.

Preparation of Fairbanks 3.5-17% continuous exponential gradient SDS PAGE gel. 3.5-17% continuous polyacrylamide gels (Fairbanks, 1971) were poured using a two chambered gradient maker (Hoefer, USA). The gradient maker was placed on a magnetic stirrer with stirrer bars in both chambers. The taps between the chambers and at the front connection to the pump were closed. 3.5% acrylamide mixture was pipetted into the back and 17% acrylamide mixture into the front chambers (table 4). The magnetic stirrer was switched on for the duration of the gel pouring. TEMED and APS solutions were added and stirred for five seconds. The front chamber was sealed with a plunger, both taps were opened and the acrylamide pumped at 2ml/min (1.5mm X 14cm X 16cm). Samples were electrophoresed at 50 volts in X1 Fairbanks buffer for 18 hours. Gels were stained with Coomassie blue and destained prior to quantitation by densitometric scanning (section 2.8.4).

Competitive spectrin-ankyrin binding assays using sucrose gradient purified spectrin dimer samples

Competitive spectrin-ankyrin binding studies of spectrin sucrose gradient purified dimers were performed. Sucrose gradient purified spectrin dimers were substituted for hSp in the competitive assay method.

Purification of spectrin dimers by isopycnic centrifugation. Spectrin dimers were purified by isopycnic ultracentrifugation on 5-20% sucrose gradients in isotonic KCl

	3.5% Acrylamide	17% Acrylamide
40% Acrylamide/1.5% Bisacrylamide	3.0ml	3.4ml
X 10 Gel Buffer	3.4ml*	0.8ml*
Water	26.9ml	1.62ml
10% SDS	680µl	160µl
25% Glycerol		2.0ml
TEMED	11µl	500µl (0.5% TEMED)
10% APS	0.4ml	20µl

Table 4.

Components for 3.5-17% Fairbanks continunous exponential gradient gels

*X10 Fairbanks buffer:

400mM Tris-HCl, pH 7.4,

200mM sodium acetate,

20mM EDTA.

Electrophoresis buffer: X1 Fairbanks buffer, 0.1% SDS.

buffer (section 2.5.3) (Bennett and Branton, 1977). To pour the gradients, an 8cm hypodermic needle was secured vertically by a retort stand in a SW-41 thin walled centrifuge tube (Beckman, USA) so that the tip reached the bottom of the tube. 10.5ml 5-20% linear sucrose gradients in isotonic KCl buffer were poured from a gradient maker into tubes under gravity through the needle. Gradients were then cooled to 4°C before being loaded.

1-2.3mg aliquots of hypotonic crude spectrin dimer extracts were made isotonic by the addition of X10 isotonic KCl buffer and made up to a final volume of 1.5ml with X1 buffer. Samples were pipetted onto the tops of sucrose gradients and centrifuged for 16 hours at 160,000g at 4°C in a SW41 rotor (Beckman, USA). Centrifugation was programmed for slow acceleration and no brake.

Following centrifugation, tubes were clamped to a gradient fraction collector in a cold room at 8°C. 0.5ml fractions were collected by piercing tubes with a hollow needle and running the sample out at approximately 0.5ml/min. The A_{280} values of fractions were measured, dimer fractions were pooled and then stored on ice. Protein yields were quantitated (section 2.8.1).

2.8 General protein methods

2.8.1 Protein concentration determinations

Concentration measurements from A_{280} values

Spectrin concentrations were determined from absorbances measured at 280nm using an extinction coefficient ε of 1.01 for a 1%(w/v) solution (Clarke, 1971).

Bradford protein determination method

Bradford protein determinations (Bradford, 1976) were performed with the Biorad Protein Determination Kit (Biorad, USA) as per the manufacturer's instructions. A series of BSA standards from 1μg to 20 μg were dispensed in duplicate into tubes and 1ml of 5X times diluted dye was added to each. Colour reactions were developed for 10 minutes and the absorbances measured at 595nm in a DU65 Spectrophotometer (Beckman, USA). A linear BSA standard curve was plotted with Quant II software (Beckman, USA). Typically 5-10μl samples of spectrin were analysed. 5μl membrane and spectrin depleted inside out vesicle samples were solubilised by the addition of 2μl of 10N sodium hydroxide prior to the addition of dye. The addition of the NaOH was found to have no effect on the standard values.

2.8.2 Solubilisation of samples for SDS PAGE

X5 solubilisation solution

50mM Tris.HCl, pH 8.0

5mM EDTA

5% SDS

25% sucrose

1% bromophenol blue

Spectrin, red cell membranes or spectrin depleted IOV samples at concentrations not greater than 1mg/ml were solubilised for SDS PAGE using the method of Fairbanks et al (1971). 1 volume of X5 solubilisation solution was added to 4 volumes of sample and β-mercaptoethanol was added to a final concentration of 1% (v/v). Samples were boiled for 3 minutes to solubilise proteins.

2.8.3 Staining and destaining of SDS polyacrylamide gels and non-denaturing gels

Coomassie stain solution:

0.05% Coomassie R-250 stain (BDH)

25% isopropanol

10% acetic acid

This solution was stirred vigorously for 16 hours and filtered through Watman 22 filter paper (Watman, UK).

Destaining solution

10% acetic acid

10% methanol

Gels were stained for 12-18 hours in 200ml of stain and destained with multiple changes of destain solution. It was necessary to clear the background stain for accurate densitometric scanning of gels.

2.8.4 Densitometric scanning of gels

Destained gels with a clear background were scanned using a Hoefer GS 300 Gel Scanner (Hoefer, USA) attached to an IBM compatible personal computer. Scans were recorded by the computer and peak areas integrated using GS365w software (Hoefer, USA). Gels were soaked for 30 minutes in 60% glycerol solution prior to scanning to prevent them drying out on the scanner bed. To improve scans, a piece of paper towelling was fashioned into a brush, dipped into 60% glycerol solution and run over the region of gel to be scanned. This improved the quality of the scans by making the coating of glycerol uniform and removing dust from gel surfaces.

3. Results and discussion of protein analysis

3.1 Results

3.1.2 Spectrin dimer purification

7-10mg of crude spectrin dimer extracts at concentrations between 1.5-2mg/ml were obtained from 50-60ml control and the probands' blood samples. Size exclusion column purification yielded 5-6mg dilute spectrin dimers at concentrations of approximately 0.1mg/ml. Figure 6 shows the A_{280} elution profile of P2 spectrin from a size exclusion chromatography column and details the elution of the spectrin oligomer fractions. The A_{280} profile in figure 6 was typical of the method.

3.1.3 Urea denaturation and ion exchange chromatographic separations of spectrin monomers

Initial ion exchange chromatographic separations of spectrin monomers from urea denatured crude spectrin extracts resulted in the co-elution of the band 5 and 4.1 proteins with β spectrin (results not shown). Spectrin dimers were therefore purified by size exclusion chromatography to overcome this problem. The spectrin dimer samples were bound to the ion exchange column without a concentration step to avoid yield losses on dialysis membranes. Size exclusion column buffers were prefiltered through 0.22μm filters to allow direct loading of the spectrin dimers onto the ion

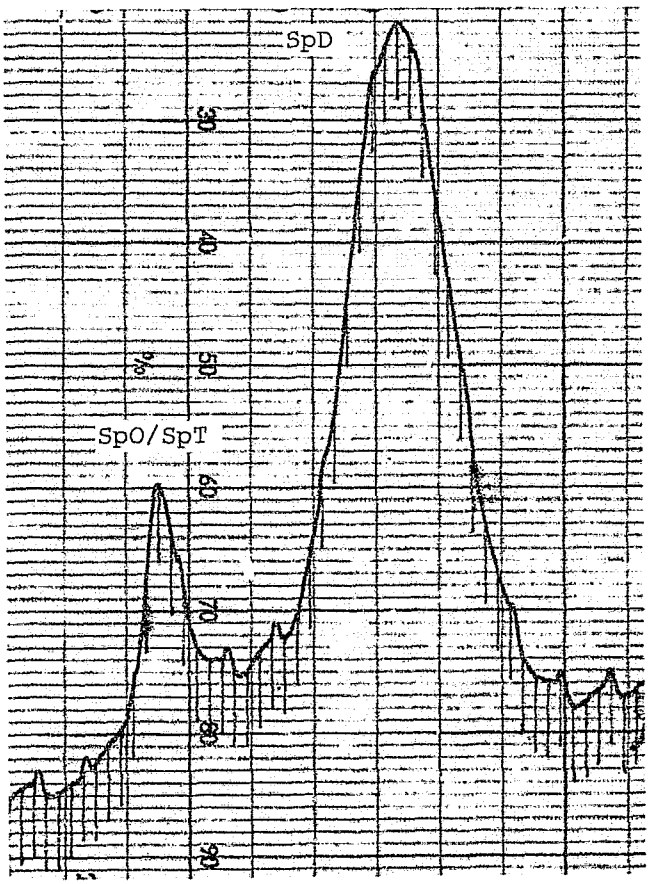


Figure 6.

Size exclusion chromatographic separation of spectrin oligomers. Shown is a typical A_{280} elution profile of spectrin oligomers from a Sepharose Cl4B column (90cm X 3cm) (Pharmacia, Sweden) in 20mM Tris-HCl, 1mM EDTA, 10mM β-mercaptoethanol. An initial 10mg of crude hypotonic P2 spectrin dimer extract in a volume of 5ml was loaded onto the column. Previous characterisation of the column with control samples showed that high molecular weight spectrin species(SpO) and tetramers (SpT) eluted in the first peak. The high molecular weight species appeared first in the void volume (approximately 90ml) followed by SpT in the same peak. Pure spectrin dimers (SpD) eluted in the second peak. In the preparation shown, 4ml fractions (denoted by tick marks) were collected at a flow rate of 0.4ml/min and pooled. 7mg of pure spectrin dimers at a concentration of 0.1mg/ml were recovered.

exchange column.

Figures 7, 8, 9 and 10 and table 5 detail the results of spectrin monomer preparations. 5mg aliquots of control and P2 size exclusion chromatography column purified spectrin dimer were denatured in urea and loaded onto an ion exchange chromatography column. The A_{280} profiles of sodium chloride elutions of control and P2 spectrin subunits from the ion exchange column are shown in figures 7A and 8A. Two protein peaks appeared, (i) between 280mM and 350mM sodium chloride and (ii) between 350-400mM sodium chloride. The monomer separations were performed in batches on the same day with the same solutions. The elution profiles shown are typical of the results obtained for both probands and there were no significant differences between separations using control or the probands' spectrin.

SDS PAGE of pooled control and P2 peak (i) fractions and selected peak (ii) fractions are shown in figures 7B and 8B. Pooled peak (i) fractions contained pure β-spectrin. The second protein peak contained predominantly α spectrin with varying amounts of β spectrin contamination. Table 5 gives the relative amounts of α and β spectrin, measured from densitometric scans, in peak (ii) fractions of a control and P2 spectrin monomer separation. From these results, α Spectrin fractions from peak (ii) with the lowest β spectrin contamination were chosen and pooled. In this preparation the contamination was maximal at 20% of the total α+β spectrin.

In figure 9 the SDS PAGE separations of measured aliquots of the pooled control and

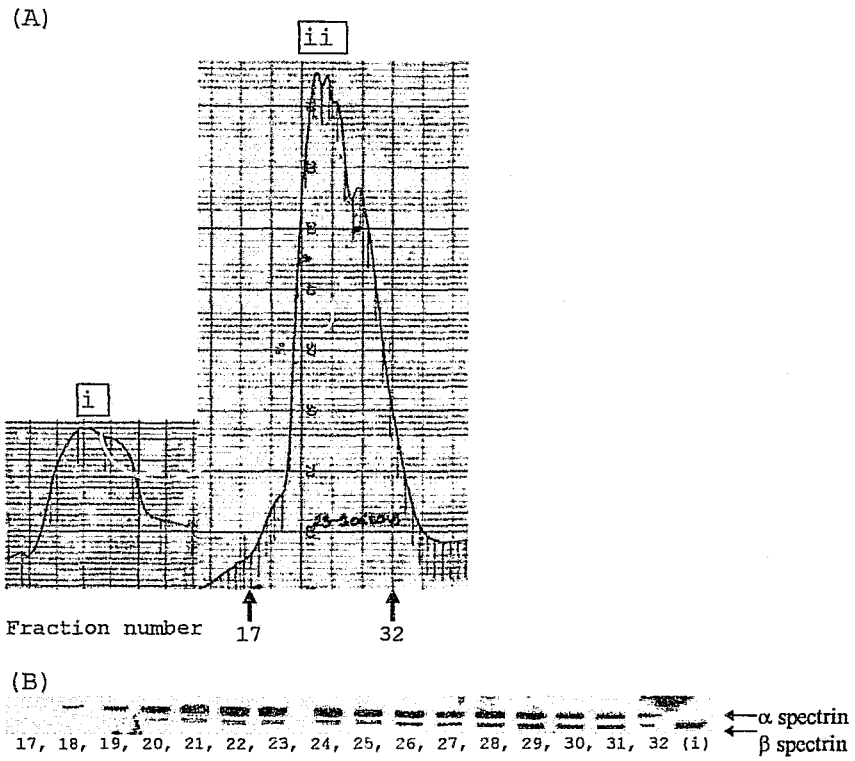


Figure 7.

Control spectrin subunit elution from a MonoQ ion exchange chromatography column and SDS PAGE separations of column fractions. (A) Typical A_{280} profile of a NaCl gradient elution of control α and β spectrin subunits from a MonoQ (Pharmacia, Sweden) ion exchange chromatography column. Protein peak (i) eluted between 280-350mM NaCl and was collected in 2ml fractions denoted by the tick marks. Peak (ii) eluted between 350-400mM NaCl and was collected in 0.5ml fractions. The recorder sensitivity was doubled for the collection of peak (ii). (B) 10μL aliquots of selected fractions were separated on 8% SDS PAGE discontinuous gels. The positions of the selected fractions are indicated below peak (ii). Gel lane (i) is an aliquot of pooled peak (i). Protein peak (i) contained pure β spectrin and peak (ii) fractions contained α spectrin contaminated with β spectrin.

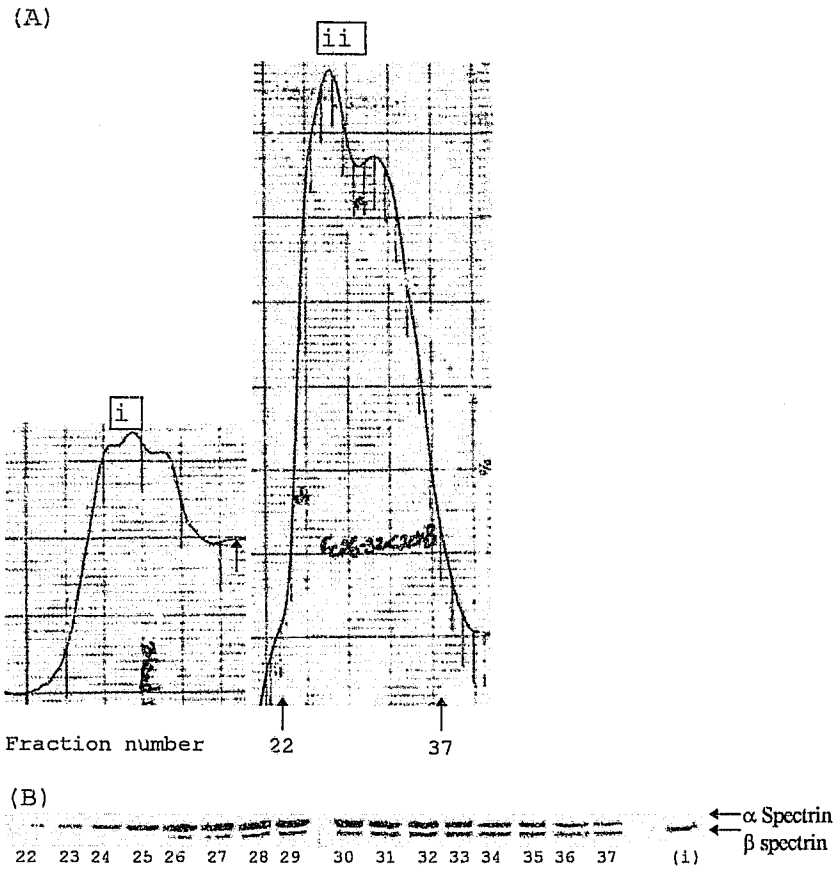


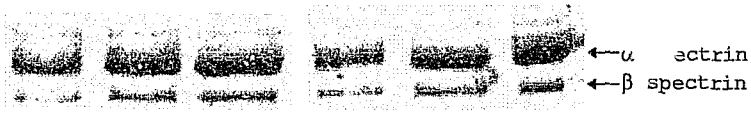
Figure 8.

Elution profile of P2 spectrin subunits and SDS PAGE separations of fractions. (A) A_{280} profile of a NaCl gradient elution of P2 α and β spectrin subunits from a MonoQ (Pharmacia, Sweden) ion exchange chromatography column. Protein peak (i) eluted between 280-350mM NaCl and was collected in 2ml fractions denoted by tick marks. Peak (ii) eluted between 350-400mM NaCl and was collected in 0.5ml fractions. The recorder sensitivity was doubled for the collection of peak (ii). (B) SDS PAGE of selected fractions. Fractions numbers are indicated below each gel lane. Lane (i) is an aliquot of pooled peak (i). Protein peak (i) contained pure β spectrin. Peak (ii) contained α spectrin contaminated with β spectrin. The elution profile is typical and is similar to the control spectrin monomer separations shown in figure 6.

Control fraction	% β spectrin		Proband 2 fraction	% β spectrin
22	25		26	20
23	12		27	14
24	11		28	12
25	11		29	13
26	16		30	16
27	14		31	11
28	14		32	22
29	19		33	29
30	24			

Table 5.

Relative amounts of α and β spectrin in α spectrin fractions eluted on a sodium chloride gradient from a MonoQ ion exchange chromatography column. Control and P2 α spectrin fractions eluted from a MonoQ ion exchange column (Pharmacia, Sweden) on a 350-400mM sodium chloride gradient were analysed by densitometric quantitation of SDS PAGE separations. The percentages of α and β spectrin in each lane were calculated from the total (α + β) spectrin. Control fractions 23-29 and P2 fractions 26-32 were pooled. These results are typical of the results obtained for this separation.



Lane	1	2	3	4	5	6
OD units/ lane	2	3	4	2	3	4
%β spectrin	12	15	13	21	19	21

Figure 9.

SDS PAGE gels of pooled control and P2 α spectrin fractions separated by ion exchange chromatography. Spectrin subunits were separated by ion exchange chromatography and α Spectrin fractions analysed. Fractions with β spectrin contamination below 20% were pooled and pooled samples were then separated by SDS PAGE. The relative levels of α and β spectrin were measured by densitometric scanning. Lanes 1-3 and 4-6 show SDS PAGE separations of pooled control and P2 α spectrin fractions respectively. For comparison, 2, 3 and 4 absorbance units (A_{280} X volume in μL) of each sample were analysed. The percentage β spectrin in each sample, expressed as a percentage of α+β is given. There is no significant difference between measurements taken from 2-4 absorbance units of each sample.

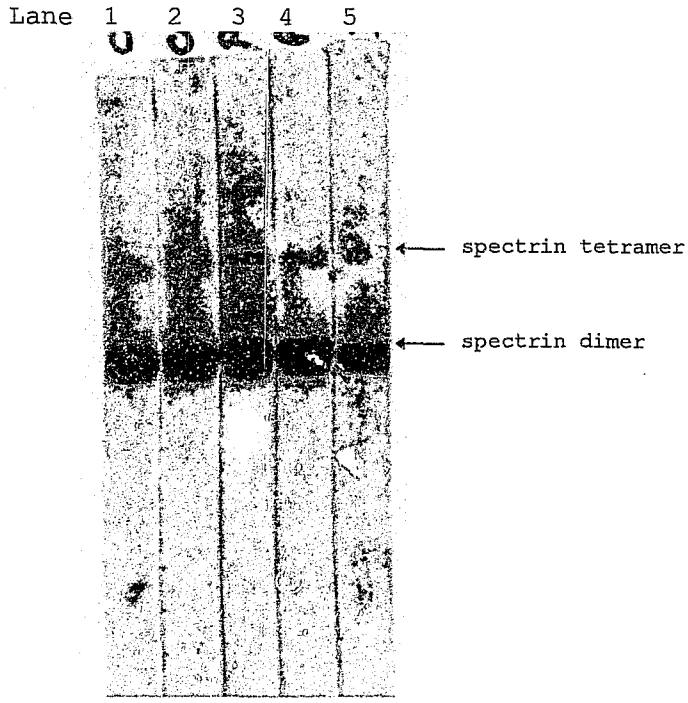


Figure 10.

Non-denaturing gel separations of hybrid spectrin oligomers. The oligomeric states of hybrid spectrins comprising control (C_α and C_β) and P2 (P_α and P_β) spectrin subunits were analysed on non-denaturing gels. Lanes: (1) $C_\alpha C_\beta$, (2) $C_\alpha P_\beta$, (3) $P_\alpha C_\beta$,(4) $P_\alpha P_\beta$ and (5) purified spectrin dimer. The hybrid spectrins were predominantly in the dimeric form with some tetramer present. In this preparation there was no evidence of high molecular weight species.

P2 α spectrin fractions detailed in table 5 with the relative amounts of monomer in each are shown. Equal loadings based upon the A_{280} value and volumes of control and P2 samples were analysed. There was an average of 13% and 20% β spectrin contamination in the control and P2 samples respectively. There was no significant difference between the measurements of β spectrin contamination for loadings of pooled sample between 2-4 absorbance units. For this reason 2.5 absorbance units of pooled α spectrin samples were routinely analysed in subsequent preparations. α Monomer yields, calculated from the volume multiplied by the A_{280} value, were in a large excess to the β spectrin.

An overview of the results of several preparations shows that, after renaturation, hSp yields were between 50-250μg at concentrations between 0.12-0.23mg/ml. Major losses were incurred by the adsorption of monomers and hSp to dialysis membranes during the renaturation and concentration steps. Preparations lasted five days and despite attempted standardisation, large variations in yields between batches and within batches occurred.

3.1.4 hSp renaturation

The renaturation conditions produced predominantly hSp dimers with some tetramers as shown in figure 10. In the gels shown there is no evidence of high molecular weight species. This was not always the case as there were preparations where high molecular weight species occurred. Attempts at further purification of hSp dimers using sucrose

gradient centrifugation resulted in almost complete yield loss and renatured hSp were used without further purification.

3.1.5 Analyses of proband spectrin structure

Isotonic crude spectrin dimer extracts from control, the probands and the mother were digested with trypsin under mild isotonic conditions. Tryptic peptide maps were produced by separating digests on a two-dimensional system incorporating IEF and SDS PAGE.

Tryptic peptide maps of control, the mother's and the probands' spectrin are shown in figure 11; peptides are labelled according to the nomenclature of Speicher and Marchesi (1982).

A comparison of the P1 and P2 digest peptide maps with the control shows similar alterations of spectrin αII and αIII domain peptides in both probands. The 46kDa αII domain peptide showed an acidic shift in pI; best seen by viewing the position of the αII peptide relative to the αI and βIV peptides. The shift was estimated to be 0.1pH units from a pH gradient measured from a blank IEF gel (results not shown). In addition to the pI shift, the αII domain peptides at 30kDa and 35kDa are absent in the probands' digests. The 25kDa αII peptide appears to be normal. The 52kDa αIII peptide present in the control is absent in the probands. This change in the αIII domain 52kDa peptide has been observed in control subjects (Private communication T Coetzer and Coetzer et al, 1985). No other changes in the pI values or molecular

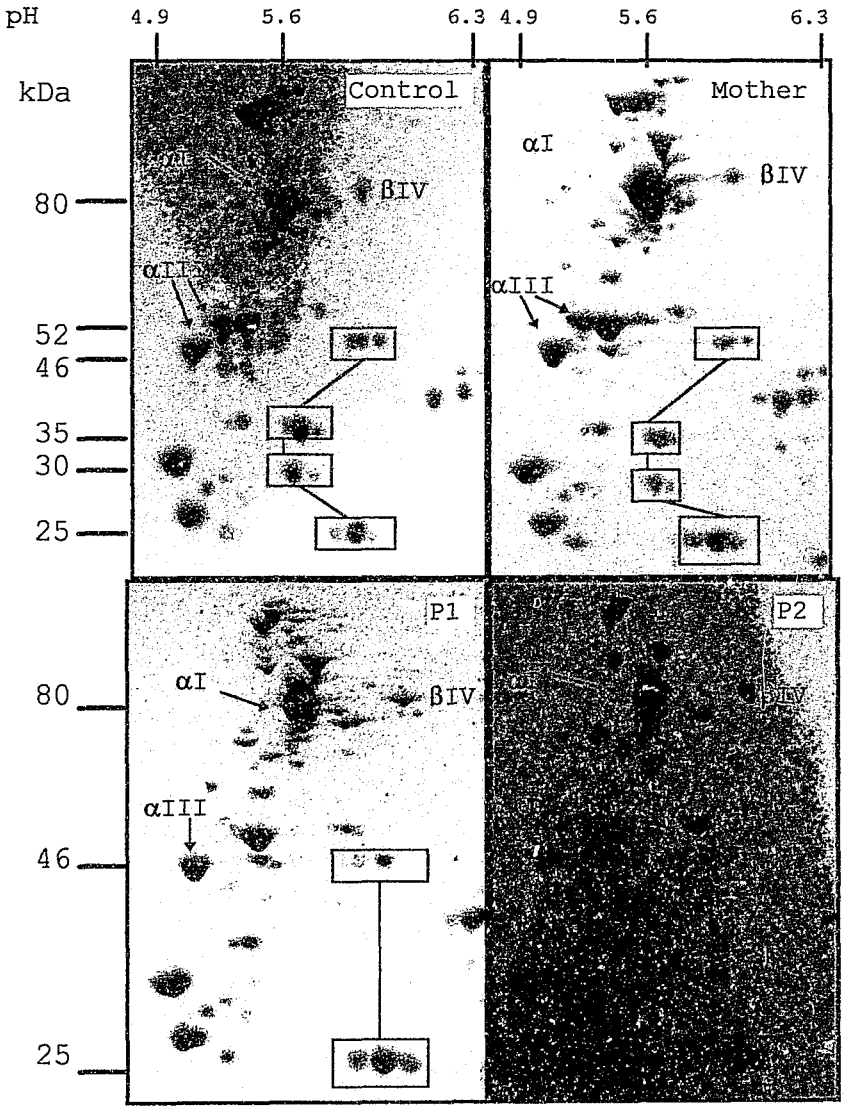


Figure 11.

Two dimensional spectrin tryptic digest peptide maps. Control, the mother's, P1 and P2 spectrin extracts were digested with trypsin under mild isotonic conditions at 0°C. Tryptic digest peptides were separated according to isoelectric point (horizontal separation) and molecular weight (vertical separation). Structural domains are indicated using the nomenclature of Speicher and Marchesi (1982). αII Domain peptides are outlined in boxes

and spectrin from both probands showed the same alterations of this domain: an acidic shift of approximately 0.1pH units in the pI of the 46kDa peptide and an absence of the 30kDa and 35kDa peptides. The 52kDa αIII peptide was also absent in the probands' digests. The mother's spectrin tryptic digest was normal.

weights of other digest peptides were observed. These digest data are similar to that reported for spectrin St Claude (LeComte et al, 1990a and Fournier et al, 1997).

The peptide map of the mother's spectrin digest is the same as the control except for a reduction in the intensity of the αIII 52kDa peptide. A tryptic digest of the father's spectrin was also found to be the same as the control (S. Lambert private communication).

The spectrin αII changes were not observed in a study of 40 white and 40 black control subjects nor in 14 kindreds with common HE that included 5 kindreds with partial protein 4.1 deficiency and one patient with an abnormal αI domain and spectrin deficiency (Private communication, S. Lambert and T.L. Coetzer). Therefore the altered αII domain and the functional defects of the probands' spectrin were co-incidental implying a possible causal relationship.

To investigate which proband spectrin chain was the primary cause of the α spectrin alterations seen in the probands' spectrin digests, hSp incorporating monomers from control and P1 were renatured in isotonic buffer and digested with trypsin under the same conditions used for whole spectrin. Two dimensional tryptic peptide maps of hSp digests are shown in figure 12. A comparison of the tryptic digests of $P_{\alpha}C_{\beta}$ and $P_{\alpha}P_{\beta}$ with the probands' spectrin digests in figure 11 shows them to be the same with: an acidic shift in the pI of the αII 46kDa peptide, absent αII breakdown products at 35kDa and 30kDa, an increased intensity of the αII 25kDa peptide most notable in the

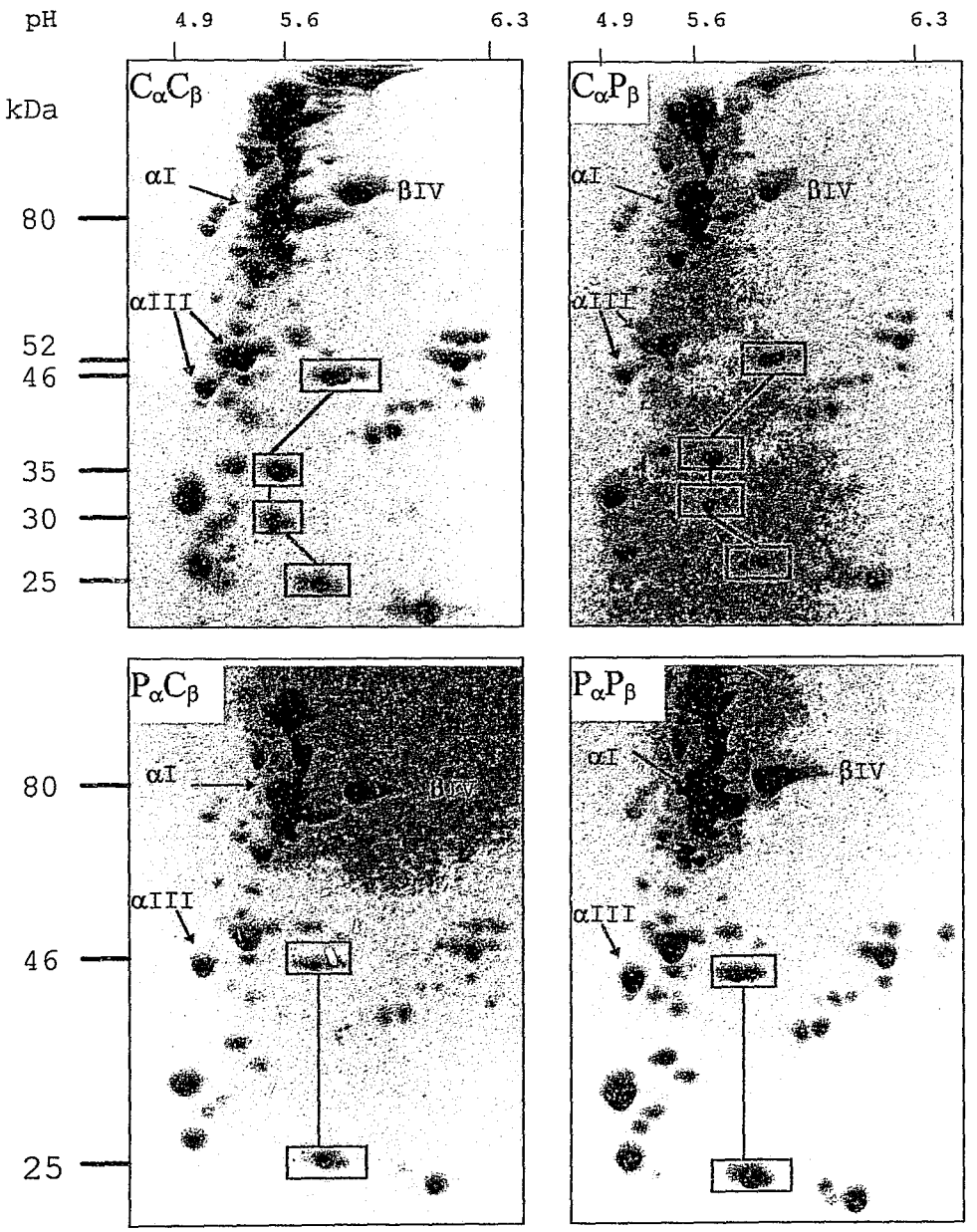


Figure 12.

Two dimensional peptide maps of hybrid spectrin trytpic digests. Tryptic digests of hybrid spectrins, reassociated from control (C_α and C_β) and P1 (P_α and P_β) spectrin subunits, were

separated by isoelectric focusing (horizontal) and SDS PAGE (vertical). The major αI, αII and βIV peptides are indicated. pH values were ascertained for specific digest peptides from a previous control spectrin digest. Hybrid spectrins reassociated with C_α are normal. The αII domain peptides, outlined in boxes, in digests of the hybrid spectrins incorporating the P_α chain show an acidic shift in the pI of the 46kDa αII peptide and an absence of the 30kDa and 35kDa peptides. The αIII domain 52kDa peptide, indicated by an arrow, is also absent in these hybrid spectrins.

$P_\alpha P_\beta$ digest and absent 52kDa αIII peptide. The molecular weights and pI values of the $C_\alpha P_\beta$ and $C_\alpha C_\beta$ tryptic digest peptides were similar to the control digest in figure 11. A tryptic peptide map of isolated P2 α spectrin shown in figure 13 shows the same changes in the αII and αIII peptides as the $P_\alpha C_\beta$ and $P_\alpha P_\beta$ hSp digests in figure 12 and whole P2 spectrin digest in figure 11.

The digest data thus demonstrated that the altered proband spectrin αII and αIII domain peptides were limited to hSp formed with proband α spectrin. The cause therefore was a defective α spectrin chain with no contribution from β spectrin.

3.1.6 hSp dimer self-association assays

hSp dimers were self-associated into tetramers by incubation at 30°C. This was to identify the proband spectrin subunit causing the spectrin dimer self-association defect (Coetzer and Zail, 1982).

hSp comprising subunits from control and P2 were concentrated for dimer self-association assays by dialysis against PEG which resulted in large yield losses. Enough $C_\alpha C_\beta$, $C_\alpha P_\beta$ and $P_\alpha P_\beta$ at concentrations equal to or above 0.5mg/ml was produced for triplicate assays. Only enough $P_\alpha C_\beta$ at a concentration of 0,3mg/ml was available for a single measurement at 0.3mg/ml and duplicate $C_\alpha C_\beta$, $P_\alpha C_\beta$ and $P_\alpha P_\beta$ assays were also performed at 0.3mg/ml.

A comparison of the average initial and final levels of hSp tetramers in table 6 shows

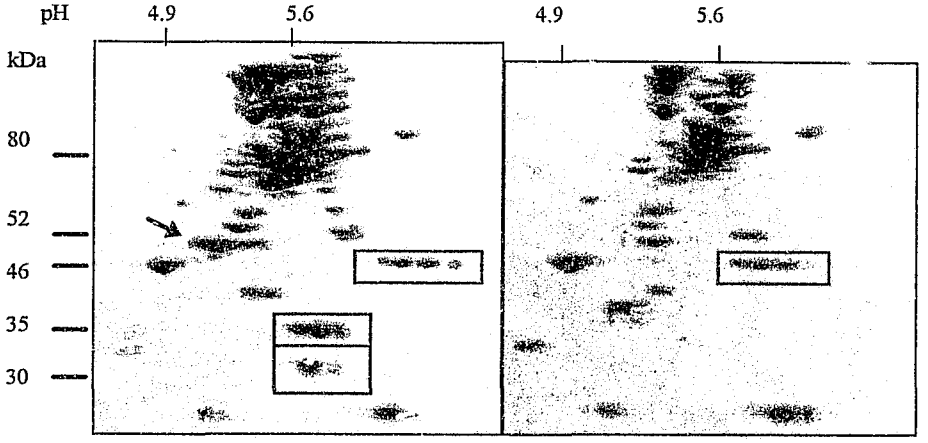


Figure 13.

Two dimensional peptide maps of control and P2 α spectrin tryptic digests. Isotonic samples of control and P2 α spectrin monomers were digested with trypsin under mild conditions. Tryptic peptides were separated in two dimensions according to isoelectric point and molecular weight. In the P2 digest: the 46kDa αII domain peptide (green boxes) showed an acidic shift and the 30kDa and 35kDa αII peptides (red boxes in the control) were absent. The P2 52kDa αIII peptide was also absent (arrow in the control).

hSp	$C_\alpha C_\beta$	$C_\alpha P_\beta$	$P_\alpha P_\beta$		$C_\alpha C_\beta$	$C_\alpha P_\beta$	$P_\alpha C_\beta$	$P_\alpha P_\beta$
mg/ml hSp	0.5	0.5	0.5		0.3	0.3	0.3	0.3
initial % hSp tetramer	14.8	12.3	19.2		18.9	14.3	19.0	13.6
final % hSp tetramer	50.4	48.4	32.8		41.5	38.9	24.3	20.7

Table 6.

hSp dimer to tetramer conversion assays. hSp, formed from control (C_α and C_β) and P2 (P_α and P_β) monomers, at concentrations of 0.5mg/ml and 0.3mg/ml were incubated at 30°C for 6 hours to convert hSp dimers to tetramers. Equilibrium levels of dimers and tetramers were quantitated by scanning densitometry of non-denaturing gel separations of samples. The relative proportions of dimers and tetramers are expressed as a percentage of the total dimers and tetramers in a lane. hSp reconstituted with the P_α chain showed reduced self-association.

that tetramers were increased in the four hSp by 30°C incubation. Figure 14 indicates that the increased percentage of tetramer levels did not result from dimer aggregation as no high molecular weight spectrins were formed during the 30°C incubation. This demonstrates that the dimer self-association function was regained by the renatured hSp.

The dimer self-association assays measured equilibrium levels of spectrin oligomers (Ungewickel and Gratzer, 1978) and determined the abilities of hSp dimers to self-associate. Tetramer formation was reduced in hSp containing the P_α spectrin chain and was independent of the P_β spectrin. SDS PAGE separations of the hSp after incubation at 30°C, figure 15, indicated that the samples consisted of pure spectrin monomers which were not proteolysed before or during the 6 hour 30°C incubation. Therefore the reductions seen in the self-association of $P_\alpha C_\beta$ and $P_\alpha P_\beta$ were not artefacts attributable to proteolysis.

The assay data therefore identified a defective proband α spectrin as the cause of the probands' spectrin dimer self-association defect with no contribution from the proband's β spectrin subunit.

3.1.7 Ankyrin binding assays

The hSp-ankyrin binding assays aimed to identify the spectrin monomer causing an observed marked proband spectrin-ankyrin binding defect (Zail and Coetzer, 1984).

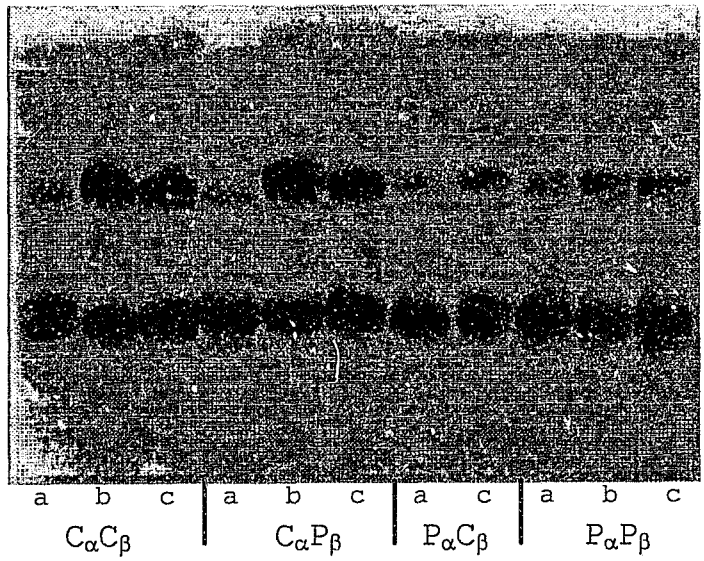
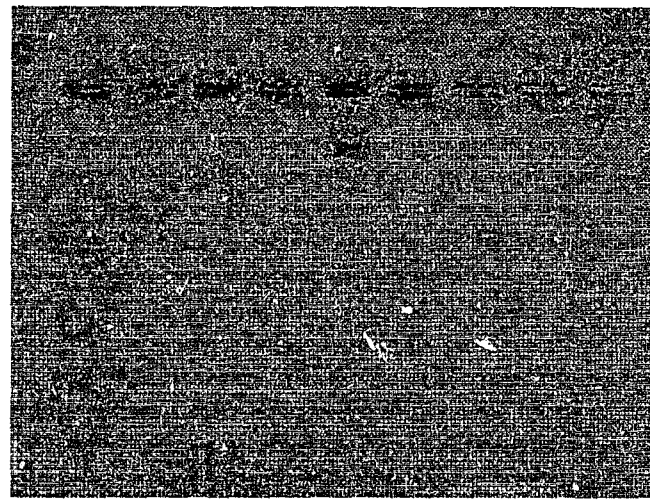


Figure 14.

Hybrid spectrin dimer self-association assays. Hybrid spectrin dimers, formed from control (C_α and C_β) and P2 spectrin monomers (P_α and P_β), were converted to tetramers by incubation for 6 hours at 30°C. Hybrid spectrin oligomers were separated on non-denaturing gels. Gel series **a** are hybrid spectrins sampled prior to incubation and series **b** and **c** are assays of hybrid spectrins at concentrations of 0.5mg/ml and 0.3mg/ml respectively after incubation at 30°C for 6 hours.



(A) (B)

$C_\alpha C_\beta$ $C_\alpha P_\beta$ $P_\alpha C_\beta$ $P_\alpha P_\beta$ MW $C_\alpha C_\beta$ $C_\alpha P_\beta$ $P_\alpha C_\beta$ $P_\alpha P_\beta$

Figure 15.

SDS PAGE of hybrid spectrins used in dimer self-association assays. Hybrid spectrin dimer samples comprising control (C_α and C_β) and P2 (P_α and P_β) spectrin monomers were converted to tetramers at 30°C for 6 hours. 1μg samples taken (A) prior to and (B) after incubation were separated by SDS PAGE. Red blood cell membrane protein molecular weight markers were electrophoresed in the lane marked MW. The spectrin samples showed no proteolysis as a result of the incubation.

^{125}I radiolabelled hSp-ankyrin binding assays

The observed ankyrin binding defect of the probands' spectrin was assayed using spectrin depleted IOV. IOV are red cell membranes treated to remove ankyrin. The free ankyrin binds added spectrin thus providing a means of quantitating the binding. Inital analysis of β spectrin-ankyrin binding was performed using monomers purified by preparative SDS PAGE (results not shown). The SDS PAGE method produced low yields of hSp in poor quality which could not be assayed. After ankyrin binding assays, the pellet radioactivity of duplicate β spectrin assays were lower than the heat denatured blank assays. Thus it appeared that the heat denaturation was causing massive aggregation of the protein to the spectrin depleted IOV . Monomers were then prepared using urea denaturation followed by ion exchange chromatographic separation. Despite this and successful hSp production, the anomalous behaviour of heat denatured blank assays seen in the β spectrin assays of SDS prepared samples persisted in the hSp. It was observed that ^{125}I labelled heat denatured hSp were not pelleted in the absence of vesicles implying that the hSp were heat labile and adhered to IOV possibly by apolar interactions. hSp-ankyrin binding was then studied using a competitive inhibition assay.

hSp-ankyrin competitive inhibition binding assays

In an attempt to overcome the problem of the heat denatured blank assays experienced with labelled hSp, hSp-ankyrin binding assays measuring the extent of inhibition of labelled control spectrin binding to spectrin depleted IOV were performed. The

omission of a hSp labelling step had the added advantages of increased hSp yields and concentrations by removing a dialysis procedure and reduced the time elapsed from initial spectrin extractions to binding assays from 10 to seven days.

In the competitive spectrin-ankyrin binding assays used, a range of amounts of hSp (0-19.5μg) competed with 1.25μg of ^{125}I labelled control spectrin dimers for ankyrin binding sites on 16μg of spectrin depleted IOV under standard conditions. Assays were performed in duplicate and heat denatured hSp blanks were included to measure the amount of non-specific hSp competition. Assays were terminated by pelleting IOV by centrifugation and the radioactivity of pellets was measured.

Table 7 shows the radioactivity counts per minute of the pelleted spectrin depleted IOV from the hSp-ankyrin competitive binding assays used to draw figure 16. In this assay, the average radioactivity measured from eight 1μg standards of ^{125}I labelled control spectrin dimers was 36280cpm. From this value, the amounts of control spectrin dimer bound to pelleted spectrin depleted IOV was calculated from the values in table 7 and expressed as μg of ^{125}I labelled spectrin dimer per mg spectrin depleted IOV protein.

To plot the curves in figure 16, the contribution to the reduction in radiolabelled control spectrin binding by the non-specific component of hSp competition in each

μg hSp	$C_\alpha C_\beta$		$C_\alpha P_\beta$		$P_\alpha C_\beta$		$P_\alpha P_\beta$	
	Ave	bl	ave	Bl	ave	bl	ave	bl
0	19902		19902		19902		19902	
5	11117	19177	11742	19031	15849	20015	18458	19897
7.5	9263	17923	9738	19176	14907	18343	17074	18820
10	8211	16472	8037	17930	14065	16602	15450	17610
15	7136	18855	6430	17861	13076	18776	13785	16967
19.5	5659	18223	5768	18901	11825	19285	11238	17936

Table 7.

Radioactivity measurements of spectrin depleted IOV bound radiolabeled control spectrin dimers from competitive ankyrin binding assays. hSp, formed by the reassociation of control (C_α and C_β) and proband 1 (P_α and P_β) monomers, competed with ^{125}I radiolabelled control spectrin dimers for ankyrin binding sites on 16μg of spectrin depleted IOV. Duplicate assays and a heat denatured hSp blank were performed. The radioactivity values were measured from IOV pelleted by centrifugation. Shown are the average (ave) and blank (bl) radioactivity counts per minute for the series of competitive hSp binding assays plotted in figure 16.

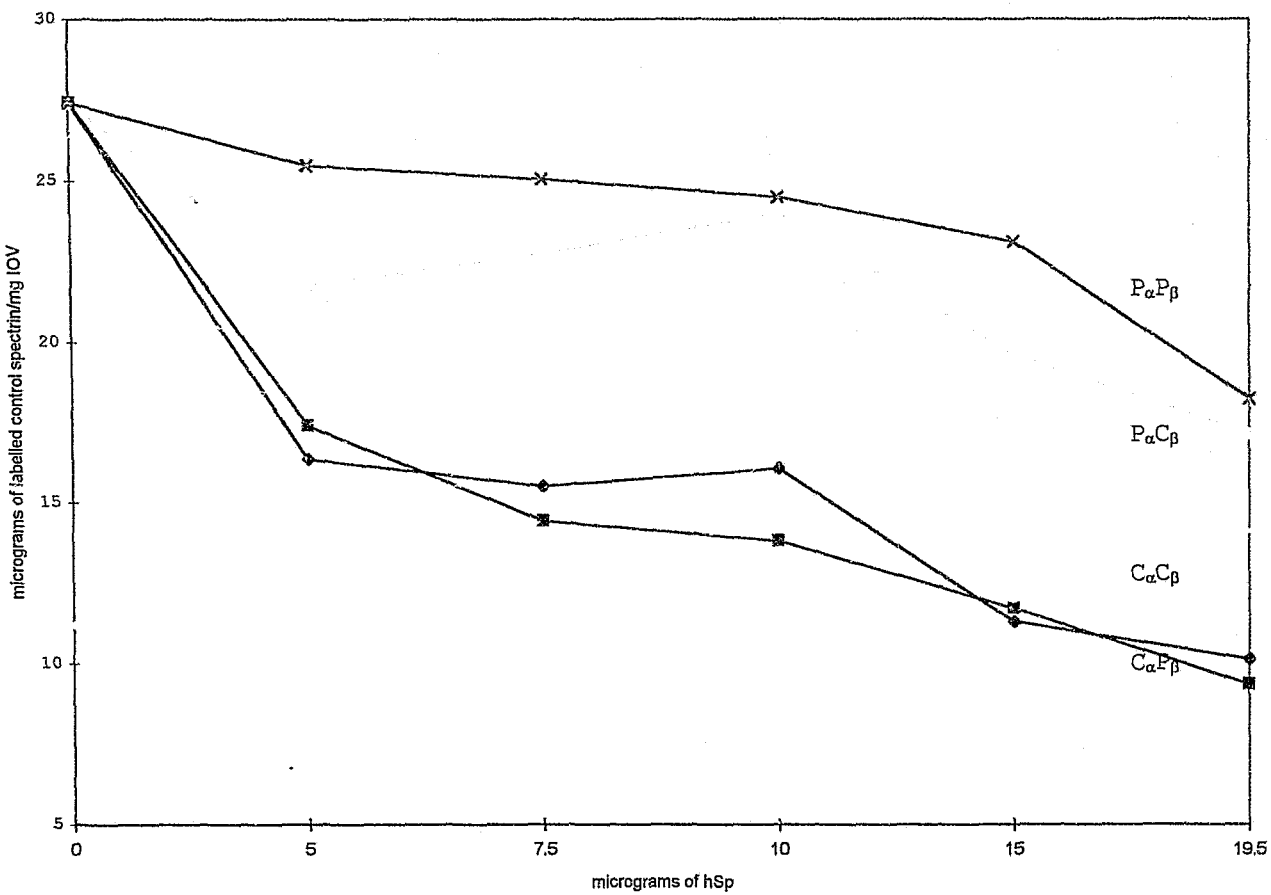

30
25
20
15
10
5
0
5
7.5
10
15
19.5
micrograms of labelled control spectrin/mg IOV
micrograms of hSp
$P_\alpha P_\beta$
$P_\alpha C_\beta$
$C_\alpha C_\beta$
$C_\alpha P_\beta$

Figure 16.

Hybrid spectrin-ankyrin competitive inhibition binding assay curves. Hybrid spectrins comprising control (C_α and C_β) and P1 (P_α and P_β) spectrin monomers were assayed for their abilities to inhibit labelled control spectrin dimer binding to spectrin depleted IOV. A range from 0-19.5μg of hybrid spectrins competed with 1.25μg of ^{125}I labelled control spectrin dimers for ankyrin binding sites on spectrin depleted IOV. The binding data are shown as the mean of duplicate points. Data ranges were less than +/- 8.5%. The curves indicate that hybrid spectrins with the P_α chain were less able to inhibit control spectrin binding than the hybrid spectrins reassociated with a C_α chain.

assay was calculated. To explain the calculation, a result from the $C_\alpha P_\beta$ competitive hSp-ankyrin binding assay in table 7 is shown as an example.

In the 19.5μg $C_\alpha P_\beta$ assay, the radioactivity values of: the 0μg hSp competitor-free assay, the average of the duplicate assays and the blank assays were 19902 cpm, 5768 cpm and 18901 cpm respectively. Using the labelled control spectrin standard value of 36280 cpm/μg and the fact that 16μg of IOV were used in each assay, the amount (μg) of bound radiolabeled control spectrin/mg IOV in the three assays listed were: 34.4, 10 and 32.5 respectively. Subtraction of the blank assay value (B_B) from the competitor-free assay value (B_0) measures the decrease in radiolabeled control spectrin binding due to non-specific hSp competition. The measured decrease in control spectrin binding due to hSp competition (B_A) is thus greater than it should be by the amount attributable to the non-specific component. Therefore the value of the non-specific component is added to the value of B_A. In summary, the calculation for the final amount of labeled control spectrin binding (B) can be expressed as:

$$B = B_A + (B_0 - B_B)$$

The binding assay curves in figure 16 plot the amounts of labelled control spectrin dimer bound to spectrin depleted IOV in the presence of increasing amounts of hSp inhibitor. From SDS PAGE it was observed that the heat denatured hSp bound to spectrin depleted IOV to a greater extent than hSp (results not shown) as was the case in assays of labelled hSp. However a value for non-specific hSp competition was measured for each competitive assay because the non-specific displacement of

radiolabelled control spectrin dimers was measured and the hSp were not labelled. The non-specific competition component was maximal at 20% of the total binding.

hSp $C_\alpha C_\beta$ and $C_\alpha P_\beta$ inhibited the binding of control labelled spectrin dimer to the same extent as shown in figure 16. Both hSp therefore competed for ankyrin binding sites with similar ability implying that the P_β spectrin-ankyrin binding function was normal. $C_\alpha C_\beta$ and $C_\alpha P_\beta$ were markedly better able to inhibit labelled control spectrin binding than $P_\alpha C_\beta$ and $P_\alpha P_\beta$. There was scatter in the $P1_\alpha P1_\beta$ data but the results showed the same trend as the $P_\alpha C_\beta$ results.

To compare the four competitive inhibition curves of the hSp shown in figure 16, the assumption is made that the hSp samples were similar. Two properties that influence ankyrin binding: the β spectrin contamination of the pooled α spectrin fractions used in the hSp reassociations and the final hSp oligomeric states, were investigated.

The β spectrin contamination of the control and P1 pooled α spectrin fractions were 9% and 11% respectively. Therefore the different ankyrin binding properties of the hSp were not due to any significant differences in β spectrin content. The levels of dimer in the hSp sampled at the time of the binding assays, given in table 8, were similar. Approximately 90% of $C_\alpha C_\beta$, $C_\alpha P_\beta$ and $P_\alpha P_\beta$ and 82% of $P_\alpha C_\beta$ were in the dimeric form. High molecular weight species were evident from the non-denaturing gels, figure 17, of $C_\alpha C_\beta$ and $C_\alpha P_\beta$. Binding assay pellet proteins were separated by SDS PAGE and the α/β spectrin ratios were quantitated by densitometric scanning;

hSp	% spectrin dimer
$C_\alpha C_\beta$	88
$C_\alpha P_\beta$	92
$P_\alpha C_\beta$	82
$P_\alpha P_\beta$	89
Control ^{125}I SpD	100

Table 8.

Non-denaturing gel analysis of the levels of dimer spectrin in hSp samples used for ankyrin binding assays. The relative levels of spectrin oligomers in hSp samples, reassociated from control (C_α and C_β) and P1 (P_α and P_β) spectrin subunits and assayed for ankyrin binding function, were measured by scanning densitometry of non-denaturing gels. hSp were sampled at the time of binding assays and the dimer levels expressed as a percentage of the total dimers and tetramers. The levels of dimeric hSp were all similar in all the samples.

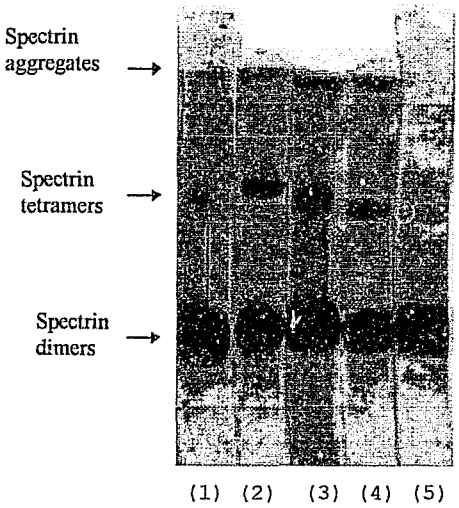


Figure 17.

Non-denaturing gel analysis of the oligomeric states of spectrin samples used for competitive ankyrin binding assays. ^{125}I labelled control spectrin dimers and hSp reassociated with control (C_α and C_β) and P1 (P_α and P_β) spectrin subunits were sampled at the time of ankyrin binding assays and analysed by non-denaturing gel electrophoresis. Gels: (1) $C_\alpha C_\beta$, (2)$C_\alpha P_\beta$ (3) $P_\alpha C_\beta$ (4) $P_\alpha P_\beta$ and (5) ^{125}I control spectrin dimer. The ^{125}I labelled spectrin was dimeric and hybrid spectrins were predominantly in the dimeric form with some tetramers present. High molecular aggregates that are visible at the tops of gels are apparent in $C_\alpha C_\beta$, $C_\alpha P_\beta$ and $P_\alpha C_\beta$.

spectrin sample	ratio of α:β spectrin
control spectrin dimer	1.1
$C_\alpha C_\beta$	0.9
$C_\alpha P_\beta$	1.1
$P_\alpha C_\beta$	0.8
$P_\alpha P_\beta$	0.9

Table 9.

α/β Spectrin monomer ratios of pelleted ankyrin binding assay spectrin depleted IOV-bound spectrins. hSp reconstituted with control (C_α and C_β) and proband 1 (P_α and P_β) spectrin subunits were assayed in competitive ankyrin binding assays. The α:β spectrin ratio of IOV bound hSp and a control spectrin dimer were quantitated by densitometric scanning of SDS PAGE gel separations of pellet proteins. The ratios indicate that the bound hSp were heterodimeric.

the results are given in table 9. The spectrin subunit ratios of $C_\alpha C_\beta$ and $C_\alpha P_\beta$ were similar to the control. The ratios for $P_\alpha C_\beta$ and $P_\alpha P_\beta$ were below the control. These ratios in conjunction with the non-denaturing gels in figure 17 indicated that the binding curves represented results for hSp heterodimers and not β spectrin homodimers. The lower ratios of $P_\alpha C_\beta$ and $P_\alpha P_\beta$ were attributable to the errors in the measurement of low amounts of the hSp bound to the pellets. The four hSp used in the ankyrin binding curves in figure 16 can therefore be directly compared. The results thus show that ankyrin binding of hSp was reduced when the P1 α spectrin chain was present. This demonstrates a novel α spectrin defect that interferes with the ankyrin binding function of β spectrin in a heterodimer.

Analysis of HS spectrin dimer-ankyrin binding

Figure 18 shows the results of a competitive inhibition spectrin-ankyrin binding assay of spectrin dimers from control and DB, an individual with HS characterised by spectrin deficiency. There were no differences between the control and the HS spectrin binding curves. Thus the spectrin deficiency observed in DB was not attributable to a spectrin binding defect.

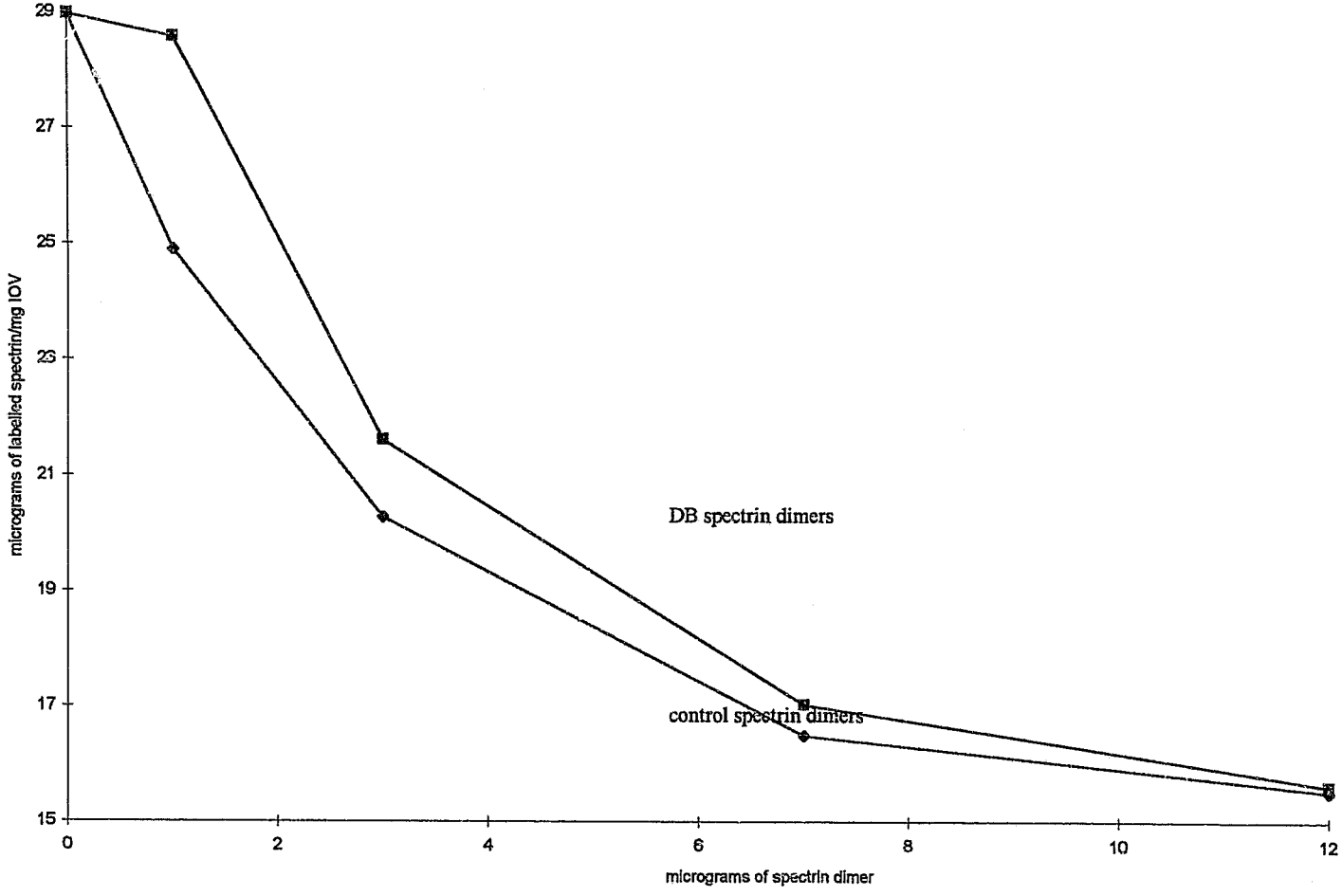

micrograms of labelled spectrin/mg IOV
29
27
25
23
21
19
17
15
0
2
4
6
8
10
12
micrograms of spectrin dimer
DB spectrin dimers
control spectrin dimers

Figure 18.

Control and DB spectrin-ankyrin competitive inhibition binding assay curves. Spectrin dimers from control (blue) and DB (red), an individual with HS characterised by spectrin deficiency, were assayed for their ability to inhibit ^{125}I-radiolabelled control spectrin dimer binding to spectrin depleted IOV. The binding data are shown as the mean of duplicate points and data ranges were less than +/- 6.4%. The control and DB spectrin showed no differences in their abilities to compete with the radiolabelled spectrin.

3.2 Discussion

3.2.1 hSp preparation

Urea was used to denature spectrin because it is a mild denaturant, can be removed by dialysis and less aggregation, in comparison to preparative SDS methods, of renatured hSp has been reported (Cohen and Langely, 1984). Additionally the urea unfolding and refolding of spectrin has been characterised (Yoshino and Marchesi, 1984 and Calvert et al, 1980b). LeComte et al (1990b) published an ion exchange method for separating spectrin monomers from an initial 5mg crude spectrin samples. Application of this method using crude spectrin dimer extracts as a starting material resulted in low levels of contaminating band 4.1 and 5 proteins in β monomer fractions. These contaminants even in small amounts can cause spectrin to form high molecular weight aggregates. This would not have affected structural assays using limited tryptic digestion but was a problem for ankyrin binding studies where the oligomeric state of the spectrin is critical. To solve this, spectrin dimers were purified by size exclusion chromatography and dilute denatured spectrin dimers were then bound to the ion exchange column before selective elution of the spectrin subunits on a sodium chloride gradient.

The major disadvantage of the ion exchange method is that α spectrin is not completely resolved from β spectrin. The ion exchange column was flushed with a large volume of buffer after β spectrin elution to remove any residual free β spectrin to improve the α spectrin purity. The β spectrin contamination of α Spectrin peak

fractions varied.. Thus the α spectrin peak was fractionated and the extent of β spectrin contamination in each sample was measured prior to pooling them. Fractions with the lowest contamination were pooled. This step was taken because upon renaturation and reassociation, the contaminating β spectrin chains form heterodimers with the α spectrin while the added β spectrin forms hSp heterodimers. Thus in a renatured hSp population there is a pool of he;erodimers that can affect the interpretation of hSp assay results and may even mask a subtle hSp defect. The β spectrin contamination in a pooled α spectrin sample had to be minimised. A guideline limit to the β spectrin contamination for the selection of α spectrin fractions for pooling was required. This had to be low enough to allow the measurement of possible subtle defects in the resulting hSp heterodimer population while yielding enough monomer to be practically useful. If an α spectrin sample has 25% β spectrin contamination, the resulting ratio of heterodimers to hSp heterodimers is 2:1 which was considered high enough for the hSp assays planned. Thus a 25% contamination level was set as an upper limit. In practice however, there was always an excess yield of α spectrin over β spectrin which allowed the pooling of α spectrin fractions with β spectrin contamination below 25%.

The measurements of the β spectrin contamination in α spectrin peak fractions were based upon SDS PAGE separations of 10μl aliquots of sample. It was not possible to predict the β monomer contamination in pooled α spectrin fractions from the initial measurements. Therefore the β subunit contamination in pooled samples was measured separately. Three

aliquots of known amounts of sample, 2-4 OD units (A_{280} X µl) were within the linear range, were analysed and an average value ascertained.

hSp were reconstituted by mixing α spectrin fractions with β monomer in a molar ratio of 1:1. Account was taken of the β spectrin contamination in pooled α spectrin fractions. To calculate the amounts of spectrin monomers for reassociation the β subunit contamination of pooled α spectrin samples was accounted for.

The details of the urea denaturation and ion exchange monomer preparations presented in the results illustrate some general features of this method: pure β spectrin was eluted from the ion exchange chromatography column in the first peak, the second peak fractions contained α spectrin with β monomer contamination, the lowest β spectrin contamination occurred in the first 20 fractions of the α spectrin peak and only these were routinely analysed.

The four hSp possible from recombination of control and a proband's spectrin monomers had to be directly compared in structural and functional assays. The inevitable variations in time and conditions that arose between monomer preparations meant that the four hSp had to be produced in parallel. This was achieved by the method described with enough yield of the hSp in suitable oligomeric states for structural and functional assays.

hSp renaturation conditions produced predominantly dimeric samples. Some tetramers and low levels of high molecular weight oligomers were evident in some preparations.

Spectrin dimers exist in either an open form capable of self-association or a closed form (Speicher et al, 1993). hSp were renatured at 8°C and at this temperature interconversion between the open and closed forms is kinetically trapped and stops spectrin tetramer formation. However within a population of renatured hSp both of these conformations would have occurred and some dimer collisions would thus have formed tetramers. Spectrin tetramer formation is a concentration dependent process (Ungewickell and Gratzer, 1978) and the low concentrations of the hSp renaturations therefore favoured dimers. Speicher et al (1993) identified a 10-20% proportion of irreversibly denatured spectrin monomers after urea treatment. There was evidence for high molecular weight species on non-denaturing gels which may have been caused by the aggregation of this permanently denatured fraction. In the method used, yields of hSp were not large enough for subsequent purification steps and any irreversibly denatured spectrin remained in the samples.

3.2.2 Functional and structural assays

The reduced dimer self-association (Coetzer and Zail, 1981), ankyrin binding (Zail and Coetzer, 1984) defect and altered tryptic digests of the probands' spectrin are features that encompass both α and β spectrin domains (Kennedy et al, 1991, Tse et al, 1990 and Speicher et al, 1993). α and β spectrin subunits closely associate in the heterodimer via non-covalent interactions (Morrow et al, 1980 and Speicher et al, 1992) which allow a primary defect in one chain to be the cause of an observable

change in the other. It was not therefore possible to ascertain unambiguously from the spectrin data which proband monomer was abnormal. Assays of hSp comprising control and proband monomers were therefore conducted to identify the defective spectrin monomer causing a particular defect. These assays identified an defective proband α spectrin chain.

Tryptic cleavage of spectrin is a means of probing its structure (Speicher and Marchesi, 1982). The observed alteration of the probands' spectrin αII domain tryptic peptide maps pattern therefore indicated altered structure. This change in the αII domain did not fit into the classification of polymorphisms defined by Knowles et al (1984) and its presence correlated with the functional defects of the probands' spectrin. Common to the 46kDa, 35kDa, 30kDa and 25kDa αII domain peptides is a tryptic cleavage site at α spectrin amino acid 680 (Di Paolo et al, 1993) which is shown in figure 19. The αII domain peptide series is then derived from cleavage at amino acids 1046, 981, 939 and 916 respectively. The probands' αII 25kDa peptide showed normal pI and molecular while the 46kDa peptide showed an acidic pI shift. This implied that there was a mutation between amino acids 916-1046 that either removed or disrupted the 30kDa and 35kDa tryptic cleavage sites at amino acids 939 and 981.

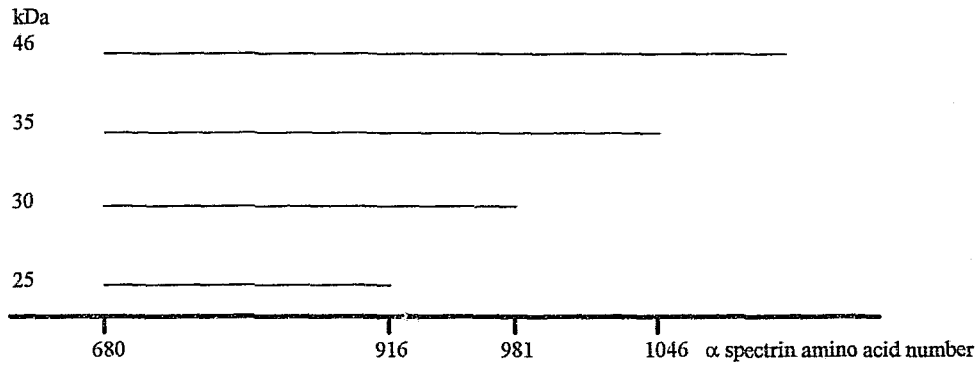


Figure 19.

Schematic representation of the tryptic cleavage sites of the α spectrin domain II 46kDa, 35kDa, 30kDa, and 25kDa peptides (represented by thin lines). The αII domain peptides are derived from a common tryptic cleavage site at amino acid 680 (amino acid position in α spectrin represented by thick line) and the peptide series is then derived from subsequent cleavage at positions towards the C-terminal. In tryptic digest maps of P1 and P2 spectrin there was an acidic shift in the 46kDa peptide, the 35kDa and 30kDa peptides were absent and the 25kDa peptide appeared to have normal molecular weight and pI value. This implied that an alteration of the amino acid sequence lay between the tryptic cleavage sites at 981 and 1046. Diagram adapted from DiPaolo et al, 1993.

The hSp functional assays indicated that an abnormal proband α spectrin reduced dimerself-association and severely impaired the ankyrin binding. This defective α spectrin was named Spectrin Johannesburg (Burke et al, 1997b), in keeping with the convention of naming abnormal spectrins after their place of discovery.

There was no evidence for a contribution to the ankyrin binding defect by an abnormal proband β spectrin. However in light of the father's mild ankyrin binding defect (Zail and Coetzer, 1984) the possibility was not completely ruled out. Thus Spectrin Johannesburg interacts with β spectrin via long range interactions to cause abnormal β spectrin-ankyrin binding. This result is further evidence for communication between the spectrin subunits within a heterodimer. Similar situations, though reversed, occur where β spectrin C-terminal point mutations cause altered α spectrin ($\alpha^{I/74}$) digestion patterns (Sahr et al, 1993, Gallagher et al, 1992, Tse et al, 1990).

In addition to the assays of P1 and P2 spectrins, spectrin dimers from an individual with HS, marked by spectrin deficient erythrocyte membranes, were analysed for ankyrin binding. There was no difference between the control and the HS patient's spectrin-ankyrin binding. Thus the spectrin deficiency in the subject's membranes were not caused by defective ankyrin binding.

A secondary goal of the assays of DB spectrin was to asses the utility of competitive inhibition spectrin-ankyrin binding assays (Zail and Coetzer, 1984) for screening multiple spectrin samples. This was considered because in a competitive assay a single control

spectrin dimer sample is radiolabelled and the test spectrin dimers are purified in tandem by density centrifugation on sucrose gradients. This is in contrast with assays where each sample is purified by size exclusion chromatography, concentrated and radiolabelled for assay (Bennet and Branton, 1977). The competitive assays provide a suitable convenient screening method for several spectrin samples.

4. DNA and RNA analysis methods

4.1Materials

All reagents were of molecular biology grade and solutions were prepared in MilliQ water (Millipore, USA).

4.2 Genomic DNA extraction

Triton X-100/saline solution

0.9% saline solution

0.2% Triton X-100

Lysis solution

10mM Tris-HCl, pH 7.5,

7M urea,

0.3M NaCl,

10mM EDTA

TE Buffer

10mM Tris-HCl, pH 8.0,

1mM EDTA

Genomic DNA was extracted according to the method of Sykes (1983). Blood samples collected in acid citrate dextrose tubes (Vacutainer, USA) were centrifuged at 800g for 10 minutes at 4°C. Aspirated plasma fractions were discarded, buffy coats were collected and if necessary stored at -70°C. Buffy coats were transferred to 50ml polypropylene tubes, mixed with approximately 30ml of Triton X-100/saline solution and centrifuged for 15 minutes at 2000g. Supernatants were discarded, cell pellets were resuspended in 30ml of saline solution by vigorous shaking and recentrifuged. Saline washes were continued until white cell pellets were a light pink colour. Pellets were dispersed in a few drops of lysis solution with glass rods. Lysis buffer was added in a dropwise fashion and the pellets further dispersed. This process was repeated until pellets were suspended in 10ml of solution. 2ml 10% SDS were added before incubating samples at 37°C for 10 minutes. Samples were extracted with 5ml of DNA phenol (Biosolve, Netherlands)/chloroform (1:1 v/v). The phenol/chloroform wash was repeated and followed by a chloroform wash. The aqueous phases from the chloroform wash were transferred to 50ml conical flasks. 10ml isopropanol (-20°C storage) were added to each and DNA immediately spooled onto the end of a glass rod. Spooled DNA samples were air dried before inverting the glass rods in 1.5ml microfuge tubes and dissolving the DNA by soaking in 0.25-1ml of autoclaved MilliQ water (Millipore, USA) water for several hours. The absorbance values at 260nm and 280nm were measured. DNA yields were determined using a factor of 1 A_{260} unit per 50μg of DNA (Ausubel et al) and the quality of the preparation was determined from the

ratio of A_{260}/A_{280} where a ratio close to 2 denoted a low level of protein contamination. The integrity of genomic DNA samples was analysed by agarose gel electrophoresis where a very slow migrating diffuse band close to the gel wells indicated that the DNA was largely intact.

4.2 PCR of genomic DNA sequences

PCR mix A

1µg of genomic DNA and 0.5uM each of the coding and non-coding oligonucleotide PCR primers in a final volume of 50µl in autoclaved MilliQ water. Details of the primers used are given in tables 10 and 11.

PCR mix B

20mM Tris-HCl, pH 8.3,

1.5mM $MgCl_2$,

50mM KCl,

0.4mM of each deoxynucleotide triphosphate,

1-2.5U Taq DNA polymerase (Boehringer Mannheim, Germany).

Volumes were made up to 50µl with autoclaved water.

Genomic DNA was diluted to 0.1mg/ml in autoclaved MilliQ water and heated to 94°C for 10 minutes to denature possible Taq DNA polymerase inhibitors prior to making

5′ primer	5′-3′sequence	3′ primer	5′-3′ sequence	amplification
p1	gaattcgactggac agttcc	p71	gaaagatagtata gaaagtt	exon 1
p1	gaattcgactggac agttcc	p2	tagggtctgctct gaggcaat	exon 1
p3	cacatataagcggg gcaacat	p2	tagggtctgctct gaggcaat	exon 2
p22	ggaattcccatgaa gactgtgcttca	p23	tataatcagttaa aaccctg	exon 3
p28	tccctgctcccagt gtctg	p29	tgggacagagg agtg	exon 4
p24	atgcaagcaggagt tttga	p25	ttcttaaggataa gaaccc	exon 5
p17	ctcatctctgtata actccag	p18	agagcctaataca aagac	exon 6
p26	tttgccctgtagtg cttgct	p27	cttctagctcaac gggttag	exon 7
p50		p51		exon 8
p72	cttttaccaggatt taaga	p73	tttgccattgttt ttgggg	exon 20
p42	CTATCCGTGACGGC CTGTTA	P43	agacgacgtctgc gttcttc	αII domain
p46	TGTCCAGTTCCAGC AGCTACC	p43	agacgacgtctgc gttcttc	αII domain

Table 10.

α Spectrin oligonucleotide PCR primer sequences. Pimer pairs used for PCR amplification of α spectrin exons 1-8, exon 20 and αII domain cDNA sequences are given. Exonic sequences were amplified with flanking intronic sequences using intron sequence primers (lower case). αII domain sequences were amplified using cDNA primers (upper case).

5′ primer	5′-3′ sequence	3′ primer	5′-3′ sequence	amplicon
p57	agttgctgcctggga aagtc	p58	ttggtagcaggtggg cggc	exon 26
p33	ggagcaatgtccagg gaaca	p56	agctgcctctgcctc aagga	exon 27
p13	gcttgtttctaaaga gagac	p5	tccacctccgctcta ctcga	exon 32

Table 11.

Sequences of β spectrin PCR oligonucleotide primer pairs. Primer pairs for the PCR amplification of β spectrin exons 26, 27 and 32 are given. β Spectrin exonic sequences were amplified with flanking intronic sequences using intron sequence primers (lower case).

mix A. Enough PCR master mix A and B was dispensed into separate tubes on ice for a proposed series of reactions. Mixes A and B were aliquoted in equal volumes into thin walled PCR tubes on ice and overlaid with mineral oil (Sigma, USA). PCR tubes were placed into a Perkin Elmer 9600 (Perkin Elmer, USA) thermal cycler preheated to 94°C and cycled 30 times for 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C. Reaction products were analysed by agarose gel electrophoresis.

4.2.2 Agarose gel electrophoresis of PCR products

DNA Loading buffer

10mM Tris-HCl buffer, pH 8.0,

1mM EDTA

25% (v/v) glycerol

0.25% bromophenol blue

Tris-EDTA buffer (TEA)

40mM Tris acetate, pH 8.5,

2mM EDTA

TEA buffer was diluted from a X50 stock solution.

Preparative and analytical amounts of PCR product, restriction digests or DNA extractions were separated by electrophoresis on horizontal submarine agarose gels in TEA buffer. Agarose was dissolved by boiling in TEA buffer, 15μg of ethidium

bromide/50ml (from a 10mg/ml stock) of gel solution was added and gels were poured. A 1/10th volume of DNA loading buffer was added to DNA samples which were then pipetted into the gel wells and electrophoresed at room temperature at 100 volts. Electrophoresis times varied according to the requirements of separations. DNA bands on gels were visualised by ethidium fluorescence on an ultraviolet light Transilluminator (UVP, USA) at 320nm. Gels were photographed with a Wratten orange filter on Polaroid 667 film (Polaroid, USA).

4.2.3 Agarose gel purification of DNA

DNA molecules separated by preparative agarose gel electrophoresis were excised. DNA was eluted from excised agarose by centrifugation in a procedure referred to as spin cleaning (Dean and Greenwald, 1995). The ends of 1ml filter pipette tips (ART, USA) were cut and placed into the tops of 1.5ml microfuge tubes. The devices were referred to as spin columns.

DNA samples separated on 0.8%-1% agarose gels were excised with a scalpel blade. Excised blocks of agarose were placed onto the filters of spin columns which were tightly sealed with laboratory film. DNA was eluted from agarose by centrifugation at 10,000g for 10 minutes at 4°C in a benchtop centrifuge. Eluted DNA was ethanol precipitated and dissolved in autoclaved MilliQ (Millipore, USA) water.

4.3 Reticulocyte total RNA preparation

Total reticulocyte RNA was extracted by a modification of the method of Chomzcynski and Sacchi (1978). All reagents, of molecular biology grade, and plasticware were from stocks reserved for RNA extractions. Solutions were made using freshly autoclaved MilliQ water (Millipore, USA) or autoclaved 0.1% (v/v) DEPC (Sigma, USA) in MilliQ water. Spatulas and other equipment in contact with RNA were autoclaved or cleaned with RNAZAP (Ambion, USA). Glassware was baked for 16 hours at 200°C.

4.3.1 Solutions for total reticulocyte RNA extraction

DEPC water

0.1% DEPC (Sigma, USA) in fresh MilliQ water was stirred at 37°C for 4 hours and then autoclaved for 40 minutes.

Solution D and D*

25mM sodium citrate, pH 7.0,

4M guanidinium isothiocyanate (Fluka, Switzerland),

1% lauryl sarcosine (BDH, UK) solution in DEPC water diluted from a 10% stock solution.

Solution D components were mixed and dissolved at 64°C and could be stored for up to 3 months at room temperature. Solution D* was prepared by the addition of 360µl

of β-mercaptoethanol to 50 ml of solution D and could be stored stored at room temperature for up to 1 month.

0.9% NaCl and 2M sodium acetate, pH 4.0 were prepared in DEPC water and autoclaved

Water saturated RNA phenol at pH 4 (Biosolve, Netherlands)

70% ethanol in autoclaved DEPC water

4.3.2 Reticulocyte enrichment

Blood collected in acid citrate dextrose tubes (Vacutainer, USA) was centrifuged at 600g for 5 minutes at 4°C to enrich the reticulocyte concentration at the top of the red cell fraction. This enrichment was based upon the fact that reticulocytes are more buoyant than mature erythrocytes (Key, 1921). Plasma fractions were aspirated and discarded. Buffy coats with the top 0.5-1ml of red cells were aspirated and placed into prechilled 15ml polypropylene tubes prerinsed with chloroform, MilliQ water and 0.9% NaCl solution. Cells, resuspended in 10-12ml of ice cold saline solution, were centrifuged at 2000g for 10 minutes at 4°C. Supernatants were aspirated and the saline washes repeated twice.

4.3.3 Cell lysis

All plasticware was treated with the anti-ribonuclease agent RNAZAP (Ambion, USA) and rinsed 3 times with DEPC water immediately prior to cell lysis. Procedures were

undertaken as speedily as possible and samples kept on ice to reduce ribonuclease activity.

Saline washed red cell pellets, approximately 0.5-1ml, were frozen at -20°C for 1-24 hours. Cells were thawed at 10°C for the minimum time required and centrifuged at 2000g for 10 minutes at 4°C in a prechilled rotor. Depending on volume, supernatants were transferred to 15ml (section 4.3.4) or 50ml (section 4.3.5) polypropylene tubes on ice.

4.3.4 RNA extraction from 0.5ml red cell lysates

5ml solution D*, 0.5ml sodium acetate, 5ml phenol and 1ml chloroform were added to 0.5ml red cell lysates in 15ml tubes with vortexing between additions of each reagent. Samples were centrifuged at 2000g for 15 minutes at room temperature. 1ml aqueous phase aliquots were dispensed into 2ml microfuge tubes and re-extracted with an equal volume of phenol/chloroform (1:1 v/v). Aqueous phases were transferred to 2ml microfuge tubes and could be stored at -70°C at this stage. RNA in each tube was precipitated with 1ml isopropanol at -20°C and pelleted by centrifugation at 10,000g for 30 minutes at 4°C in a microfuge. RNA pellets were washed with 1ml 70% ethanol, repelleted and dried in a Speed Vac (Savant, USA) centrifuge under vacuum for 5 minutes at room temperature. RNA was dissolved in 20ul DEPC treated water and stored at -70°C.

4.3.5 RNA extraction from 6ml red cell lysates

Red cell lysate supernatants were combined to a maximum of 6ml per 50ml polypropylene tubes on ice. Solution D* was added to a final volume of 30ml measured using the tube graduations, 3ml sodium acetate, 15ml phenol and 2ml chloroform were added and mixed by vortexing between each addition. Samples were centrifuged at 2000g for 15 minutes at room temperature Aqueous phases were transferred to 50 ml tubes and re-extracted with phenol/chloroform. Pooled aqueous phases were dispensed into 50 ml tubes to a maximum of 15 ml/tube and RNA was precipitated with 2 volumes of isopropanol for 16 hours at -20°C. RNA suspensions were transferred to polypropylene JA-17 centrifuge tubes (Beckman, USA) that had been incubated at room temperature in solution D* for several hours. RNA was pelleted at 22,000g for 30 minutes at 4°C, pellets were washed with 25 ml 70% ethanol and repelleted. Air dried RNA pellets were dissolved in 300-600 µl DEPC water and dispensed into 2ml microfuge tubes for storage at -70°C.

4.3.6 Analysis of Total RNA

1µl aliquots of RNA were diluted 50 times in DEPC water and the A_{260} and A_{280} values measured. Total RNA yields were calculated based upon factor of 1 A_{280} unit per 40µg of RNA. The quality of the preparation was based upon the ratio of A_{260}/A_{280}; values above 1.7 and close to 2 indicated good RNA purity (Ausubel et al). Typically 6ml of blood from individuals with reticulocyte counts close to 2% yielded 5µg of total RNA.

4.3.7 Reverse transcription-polymerase chain reaction (RT-PCR) amplification of mRNA sequences

cDNA synthesis

cDNA from total reticulocyte RNA template was synthesised using Superscript II (GibcoBRL, USA) reverse transcriptase according to the manufacturer's instructions. 1-5μg total reticulocyte RNA and 250ng of random hexamer oligonucleotide primers (Boehringer Mannheim, Germany) were pipetted into PCR tubes in final volumes of 12μl. The mixture was heated at 70°C for 10 minutes and snap chilled on ice for 5 minutes. 2μl 0.1M DTT, 4μl X5 buffer (0.25M Tris-HCl, pH 8.3, 375mM KCl, 15mM $MgCl_2$) and 1μl of a solution 10mM for each deoxynucleotide triphosphate (Boehringer Mannheim, Germany) were added and solutions were incubated at 42°C for 5 minutes. 200U Superscript II reverse transcriptase (GibcoBRL, USA) were added to reactions which were incubated for a further 50 minutes at 42°C. Reactions were stopped by heating at 70°C for 15 minutes. cDNA samples were stored at 4°C.

PCR

DNA sequences were PCR amplified from cDNA templates in two stages: the reverse transcription step was followed by nested or semi-nested PCR amplifications. Primary PCR were performed essentially as described (section 4.2.1) except that reactions contained 0.1μM of each primer (detailed in table 10), were heated for 3 minutes at 94°C prior to temperature cycling and were subjected to 40 cycles at 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes. Secondary amplifications used 1-5μl of a X100 dilution of primary PCR products as a template in the place of genomic DNA (section 4.2.1).

4.4 Subcloning of PCR amplicons

The 5' termini of PCR amplicons were phosphorylated with T4 polynucleotide kinase and 3' overhangs blunt ended with T4 DNA polymerase. DNA inserts were ligated into blunt ended dephosphorylated vector and competent *E.coli* cells were transformed with the recombinant DNA. Plasmids were prepared from bacterial colony picks for DNA sequencing.

4.4.1 Phosphorylation and blunt ending of PCR amplicons

PCR amplicons were phenol/chloroform extracted and ethanol precipitated from the reaction or purified by spin cleaning in spin columns (section 4.2.3). DNA pellets were redissolved in 25ul: 70mM Tris-HCl, pH7.6, 10mM magnesium chloride, 5mM DTT by dilution of a X 10 stock buffer (Promega, USA), 1mM ATP, 200uM of each deoxynucleotidetriphosphate and 2U of T4 polynucleotide kinase (Promega, USA). The phosphorylation reaction was incubated at 37°C for 60 minutes. 2U of T4 DNA polymerase were added and DNA was blunt ended for 5 minutes at 37°C. Enzymes were inactivated for 15 minutes at 75°C. Solutions were made up to 400µl with TE buffer, phenol/chloroform extracted and ethanol precipitated.

Vector preparation

1µg of the plasmid pGem3Z (Promega, USA) was incubated at 37°C in 20µl (10mM Tris-HCl, pH 7.5, 10mM magnesium chloride, 50mM NaCl, 0.1mM DTT) and 2U of the restriction enzyme Hind II (Boehringer Mannheim, Germany). After restriction,

3U of calf intestinal alkaline phosphatase (Boehringer, Mannheim) were added and incubated at 50°C for 60 minutes to dephosphorylate the vector.

Quantitation of amplicon and vector DNA

Vector and amplicon DNA aliquots were electrophoresed on 1% agarose gels with a Mass Ladder (GibcoBRL, USA). Amounts of DNA for the ligation reactions were estimated by comparison of standard band intensities with samples.

DNA/vector ligation reaction

Ligation reactions were performed with the Rapid Ligation Kit (Boehringer Mannheim, Germany). Amplicon and vector DNA were mixed in molar ratios of 1:1 to 1:2 to a maximum of 200ng with the kit reagents and left at 37°C for 30 minutes. A replicate mock ligation was performed to check the background level of vector religation. Where possible 1µl of the restriction enzyme Hind II (Boehringer Mannheim, Germany) was added to ligation reactions. This improved the background level of vector religation. The enzyme was included when sequence analysis showed there were no HindII sites present in the product to be ligated into the vector. Reaction products were used directly for transformation into competent *E. coli* or stored at -20°C.

Transformation of E.coli cells

LB medium

10g Tryptone (Oxoid, UK)

5g yeast extract (Oxoid, UK)

10g NaCl

10ml 1M Tris-HCl, pH7.5

Components were made up to 1 litre and sterilised by autoclaving.

LB plate agar

15g of agar were added per litre of LB medium and sterilised by autoclaving. Upon cooling 80μg/ml ampicillin (Boehringer Mannheim, Germany) was added and 30ml plates were poured in sterile petri dishes.

50μl aliquots of defrosted DH5α competent cells (GibcoBRL, USA) were transferred to sterile 1.5ml microfuge tubes on ice. Excess cell aliquots were immediately snap frozen in a dry ice/acetone bath and stored at -70°C. 5μl of each ligation reaction were added to a cell aliquot which was then incubated on ice for 30 minutes, heat shocked for 20 seconds at 37°C and placed on ice for a further 2 minutes. 500μl LB medium were added to the cells and they were incubated at 37°C for 1 hour in a shaking orbital incubator at 250rpm. 50μl and 450μl aliquots of transformed cells were plated onto ampicillin LB agar plates and incubated for 16 hours at 37°C.

Plasmid minipreparations

Solution I

25mM Tris-HCl, pH 8.0

50mM glucose

10mM EDTA

Solution II

0.2N sodium hydroxide

1% SDS

This solution was freshly prepared for each plasmid minipreparation by diluting 10% SDS and 10N sodium hydroxide solutions in MilliQ water.

Solution III

5M potassium acetate

Freshly prepared for each preparation by mixing 2 volumes of 5M acetic acid and 1 volume of 5M potassium acetate.

3M Sodium acetate, pH 5.2

Ribonuclease A/TE buffer

1µl RNAase A (Boehringer Mannheim, Germany) was added per 100µl TE.

Colonies picked from agar plates were grown for 18 hours in 2ml liquid ampicillin LB cultures (80ug/ml ampicillin) for 16 hours at 37°C in a shaking orbital incubator at 200rpm.

30% glycerol subclone stocks were prepared by adding 0.5ml of cell culture to 0.5ml 60% glycerol; stocks were stored at -70°C. The balance of the 2ml growth was transferred to 1.5ml microfuge tubes. Bacterial cells were pelleted by centrifugation at

room temperature at 10,000g for 1 minute. Supernatants were aspirated and cell pellets were resuspended in 100µl of solution I. Cell lysis was at room temperature for 5 minutes. To each tube 200µl of solution II and 150µl of ice cold solution III was added and tubes were gently inverted. Samples were incubated on ice for 15 minutes and centrifuged for 5 minutes at 10,000g at room temperature. Supernatants were transferred to 1.5ml microfuge tubes and extracted with 500µl DNA phenol (Biosolve, Netherlands)/chloroform (1:1 v/v). Supernatant aqueous phases were transferred to 1.5ml microfuge tubes and 500µl isopropanol was pipetted into each. Samples were left at room temperature for 5 minutes and then centrifuged at 10,000g for 20 minutes at room temperature. Isopropanol was aspirated and DNA pellets were air dried.

DNA pellets were dissolved in 100µl ribonuclease A/TE buffer and incubated at 37°C for 1 hour. Samples were made up to 400µl with 260µl water and 40µl of 3M sodium acetate, pH 5.2. Samples were extracted with 500µl phenol/chloroform (1:1 v/v) followed by 500µl chloroform. DNA was precipitated with 1ml ethanol (-20°C storage) for 15 minutes on ice and pelleted at 10,000g for 20 minutes at 4°C in a microfuge. DNA pellets were washed with 70% ethanol (-20°C storage) and air dried.

Recombinant plasmid DNA samples were dissolved in 50µl autoclaved MilliQ water. Plasmids were screened for insert DNA by restriction of 10µl aliquots with EcoRI and HindIII (Boehringer Mannheim, Germany). Restriction digests were analysed on 1% agarose gels.

4.4.2 Nucleotide sequencing

DNA was sequenced by the chain termination method using sequencing kits supplied with T7 DNA polymerase (Amersham, UK) according to the manufacturer's instructions.

4.5 Single stranded conformational polymorphism (SSCP) analyses

The single stranded conformational polymorphism method was based upon that of Orita et al (1989).

4.5.1 Radioactive PCR amplification of genomic DNA

Genomic DNA sequences were labelled and amplified in 25µl PCR reactions using the buffer concentrations as described (section 4.2.3) except that deoxynucleotide triphosphate concentrations were 68µM and 1µl ^{32}PdATP (10µCi/ml) (NEN, USA) was added. A 347bp amplicon from primer p2/p3 (table 10) PCR amplification was restricted with HindIII into 163bp and 184bp fragments.

1µl aliquots of PCR amplified DNA were added to 9µl of 0.1% SDS, 10mM EDTA solution. 2.5µl DNA solutions were then mixed with equal volumes of sequencing stop solution (Promega, USA), heated at 95°C for 3 minutes and snap cooled on ice.

5% acrylamide (5% T, 3.5% C) SSCP gels in TBE buffer with and without 5% glycerol were poured for use in an S2 sequencing gel apparatus (GibcoBRL, USA). 1-5µl aliquots of DNA samples were loaded onto gels and electrophoresed at 30 watts

for 4 hours while cooled by a laboratory fan at room temperature or at 4°C. Gels were autoradiographed with intensifying screens at -70°C on Curix RPI film (Agfa, Germany).

Two procedures, phenol/chloroform extraction and ethanol precipitation (Ausubel et al) are alluded to commonly in this work and are explained fully.

4.6 Phenol/chloroform treatment of DNA and RNA

To remove proteins, DNA samples were treated with TE buffered phenol (Biosolve, Netherlands) and RNA samples with water saturated phenol (Biosolve, Netherlands). Sample volumes were measured and equal volumes of phenol:chloroform (1:1 v/v) mixture were added. Samples were shaken and then centrifuged. Typically samples in 1.5ml microfuge tubes were centrifuged at 10,000g for 10 minutes in a bench top centrifuge while larger volumes were centrifuged at 2000-3000g to separate the phases. Aqueous nucleic acid containing phases were transferred to clean tubes. Denatured proteins left behind precipitated at the phenol-water interface were discarded. To remove residual phenol, samples were re-extracted with an equal volume of chloroform.

4.7 Ethanol precipitation

DNA and RNA samples were precipitated at high salt concentration by the addition of pure ethanol from a -20°C storage stock. Sample volumes were measured and a tenth

of that volume of 3M sodium acetate, pH 5.2, for DNA or 2M sodium acetate, pH 4, for RNA was added and thoroughly mixed. Two volumes of absolute ethanol (-20°C storage) was added to samples which were incubated on ice or at -20°C for a minimum of 20 minutes. Precipitated nucleic acids were pelleted by centrifugation at 10,000g for 30 minutes at 4°C. Pellets were washed with 70% ethanol (-20°C storage) to remove salts and then air dried.

5. Results and discussion of DNA and mRNA analysis

hSp assays showed that the probands' defective dimer self-association (Coetzer and Zail, 1981) and ankyrin binding (Zail and Coetzer, 1984) were caused by an abnormal α spectrin. These changes correlated with an altered proband spectrin αII domain structure. This directed an investigation of the probands' mRNA and DNA sequences coding for the αII domain. In addition investigations of the probands' sequences coding for the dimer self-association site and the ankyrin binding domain were undertaken.

5.1 Results

5.1.1 Spectrin αII domain coding sequence

Spectrin αII domain coding sequences were amplified from P1, P2 and the mother's total reticulocyte RNA by RT-PCR using primers p42/p43 (table 10) followed by a semi-nested PCR of the RT-PCR reaction products using primers p64/p43. Agarose gel electrophoresis of P1 and the mother's RT-PCR products, figure 20, showed two bands with apparent sizes of 540bp as predicted and 450bp. Both bands in the P1 reaction are of similar intensities which was a consistent feature of this RT-PCR reaction and the same result was found in P2 RT-PCR products (results not shown). In the mother, the 540bp band intensity was stronger than the 450bp band.

Nucleotide sequencing of subcloned P1 RT-PCR products, figure 21, showed that the

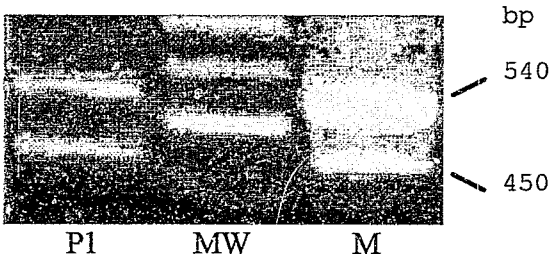


Figure 20.

Agarose gel electrophoresis of αII domain RT-PCR amplification products. αII domain sequences coding for exons 19, 20 and 21 were amplified by RT-PCR from P1 and the mother's total reticulocyte RNA. Reaction products were separated on 1% agarose gels. Reaction products from P1 and the mother (M) are shown with a 100bp ladder (Promega, USA) molecular weight marker (MW). A predicted band with an apparent size of 540bp is visible in both samples with a second smaller band with apparent size 450bp. The relative intensities of the P1 reaction products are similar; the intensity of the mother's 540bp band is greater than the 450bp band.

540bp amplicon contained a 12 base pair in-frame insertion between exon 19 and exon 20 making the amplicon 552bp in length. Exon 20 was deleted in the smaller amplicon making its exact size 447bp. The same sequences were found in P2 and no normal sequences were found in either proband.

Nucleotide sequences of subclones of the mother's 540bp RT-PCR products showed that there were two unresolved amplicons present; the normal sequence and the 12bp insertion seen in P1 and P2. Exon 20 was deleted in the 447bp fragment.

The 12bp insertion between exons 19 and 20 is in-frame and exactly matches the sequence of intron 19 directly 5' to exon 20 (Kotula et al, 1991 and P. J. Curtis, private communication). The second codon is a UAA translation stop codon which follows a CAA glutamine codon. This message is predicted to cause an α spectrin truncated at amino acid 935. The deletion of exon 20 is in-frame and is predicted to code for an α spectrin with a deletion of 31 spectrin αII domain amino acids from 935-965.

5.1.2 α Spectrin intron19/exon 20 boundary sequences

PCR amplification of α spectrin exon 20 and flanking intronic sequences from the mother's and probands' genomic DNA using primers p72 and p73 (Table 10) produced a single predicted 305bp band on 1% agarose gels and is shown in figure 22. Figure 23 shows direct nucleotide sequencing of P1 PCR product at the intron 19/exon 20 boundary. The result shows P1 is homozygous for a T⇒G mutation 13bp

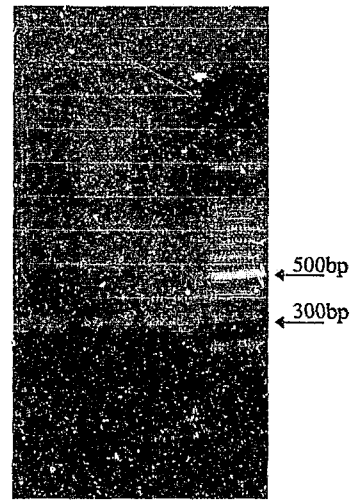


Figure 22.

Agarose gel separation of PCR amplification products of α spectrin exon 20 sequences. Exon 20 with flanking intronic sequences was amplified from P1, P2 and the mother's genomic DNA and amplification products were separated by agarose gel electrophoresis. Amplifications from P1, P2 and the mother's (M) genomic DNA all produced a single predicted 305bp amplicon. Lane 4 is a 100bp DNA ladder (GibcoBRL, USA) and the 500bp and 300bp bands are denoted by arrows.

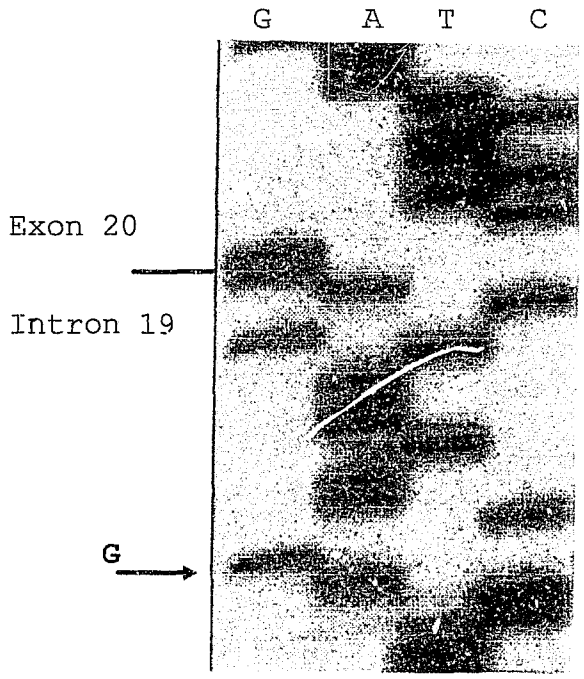


Figure 23.

Nucleotide sequence of α spectrin intron 19/exon 20 boundary in P1. The α spectrin intron 19/exon 20 boundary was amplified by PCR from P1 genomic DNA and sequenced. P1 was homozygous for an intron 19 T⇒G mutation, marked with a solid arrow, 13bp upstream from the intron 19/exon 20 boundary.

upstream from the exon 20 boundary in intron 19. Sequencing of proband 2 showed the same result and the mother was found to be heterozygous. This T⇒G change introduces an invariant 5'-ag-3' 3' acceptor splice site sequence within a polypyrimidine tract and is predicted to create a novel acceptor splice site (Padgett et al, 1986).

5.1.3 Investigation of DNA sequences coding for specific functional domains

α Spectrin dimer self association site

Spectrin dimer self-association is highly sensitive to mutations in the N-terminal αI domain of α spectrin (reviewed by Delaunay and Dhermy, 1993). Therefore the probands' α spectrin exons 1-8 were screened for possible mutations by single stranded conformational polymorphism (SSCP) analysis and α spectrin exons 1 and 2 were sequenced.

SSCP analysis uses the fact that the electrophoretic mobility of a DNA molecule in a gel is affected by its conformation. Because DNA conformation and nucleotide sequence are related; changes in electrophoretic mobility, referenced to a control, can be used to detect mutations. The DNA is denatured and single stranded DNA is electrophoresed and its electrophoretic mobility analysed. α Spectrin exons 1-8 were amplified by PCR from control, the probands' and the mother's genomic DNA using the primer sets detailed in table 10. The amplification products were all of the predicted sizes as judged from agarose gel separations (results not shown). SSCP

screening detected changes in the electrophoretic mobilities of the probands, and the mother's exon 7 PCR products relative to the control (results not shown). Nucleotide sequencing of the P1 PCR product indicated she was homozygous for a reported C⇒T mutation (Kotula et al ,1991) at codon 304 that results in a serine codon usage change from AGC ⇒ AGT. The SSCP electrophoretic mobility implied that P2 and the mother were also homozygous for the same change.

α Spectrin exons 1 and 2 and flanking intronic sequences were amplified from P1 and P2 genomic DNA using primer sets p1/p2 and p1/71 (table 10) and sequenced. Coding regions were normal but both probands were homozygous for a C⇒G mutation 4bp upstream from exon 2 in intron 1. This position in the 3' acceptor splice site consensus sequence can accommodate any base (Padgett et al, 1986) and the change was therefore predicted to be neutral.

5.1.3.2 β spectrin genomic DNA sequences

β Spectrin exon 32 was amplified with flanking intronic sequences from P1 and P2 genomic DNA using primer set p5/p13 (table 11). Nucleotide sequencing (results not shown) showed normal coding and intron-exon boundaries for both probands. The probands were however homozygous for an inserted G and deleted C 3bp and 11bp respectively downstream from the translation stop codon. These changes have been observed in other individuals (T. Coetzer unpublished observations) and were not predicted to affect the translation of β spectrin.

In light of the probands' spectrin-ankyrin binding defect, β spectrin exons 26 and 27 (Amin et al, 1993) which code for the minimum ankyrin binding domain (Kennedy et al, 1991) were amplified by PCR from P2 genomic DNA with primer sets p57/p58 and p33/p56 (table 11). Coding and intron-exon boundary nucleotide sequences were normal (results not shown). P2 was heterozygous for a reported T⇒C change (Amin et al, 1993) in exon 26. The mutation alters the leucine codon usage for amino acid 1766 from CTT to CTC and introduces a SacII restriction site. SacII restriction of amplified DNA from P1 and the mother showed that they were heterozygous for this change (results not shown).

The β spectrin gene analysis was to investigate the sequences of exons 26 and 27 coding for the ankyrin binding domain and the exons downstream to the 3' terminal exon 32. The aim was to identify any mutations responsible for the probands' spectrin-ankyrin binding defect. During this analysis however the hSp assay results identified a defective proband α spectrin chain and a normal β spectrin subunit. The protein results halted the investigation of the β spectrin gene after the sequences of exons 26, 27 and 32 were completed. The finding of normal exon 26 and 27 sequences was evidence for a long range interaction that affected the ankyrin binding domain indirectly. These results were thus consistent with the possibility of a mutant α spectrin.

5.2 Discussion

Structural analysis of the probands' spectrin directed the investigation of the probands' DNA and mRNA sequences to the region of the α spectrin gene coding for the αII domain amino acids 916-1046. The probands produce two abnormal α spectrin RNA messages: one with a 12bp insertion of intron 19 sequence and the second with exon 20 deleted. Both probands are homozygous for a T⇒G mutation 13bp uptream from exon 20 in intron 19. The defective spectrin was designated Spectrin Johannesburg and was presented in abstract form at a South African conference (Burke et al, 1997a and b) in January 1997 and at the 1997 American Society for Haematology Conference (Burke et al, 1997). During the preparation of this manuscript the same mutation was described for Spectrin St Claude (Fournier et al, 1997) in an individual from the West Indies. Erythrocyte membranes from the affected person were spectrin deficient and spectrin extracts showed mildly raised dimer levels (LeComte et al, 1990a).

Figure 24 describes the events proposed to arise from the T⇒G intron 19 mutation that produces the two abnormal RNA messages observed in the probands. The intronic mutation converts the normal pre-mRNA 5'-cuugccau-3' sequence to 5'-cuugccag-3'. This introduces an 5'-ag-3' (Padgett et al, 1986) invariant sequence for a 3' acceptor splice site at the end of a polypyrimidine (Py) tract 5'-PyPyPyPyNcag-3' sequence. The result is the creation of a novel 3' acceptor splice site. The splicing machinery recognises the novel site even though the normal intron 19 3' acceptor splice site is still present. This occurs to the exclusion of the normal intron 19 site. In

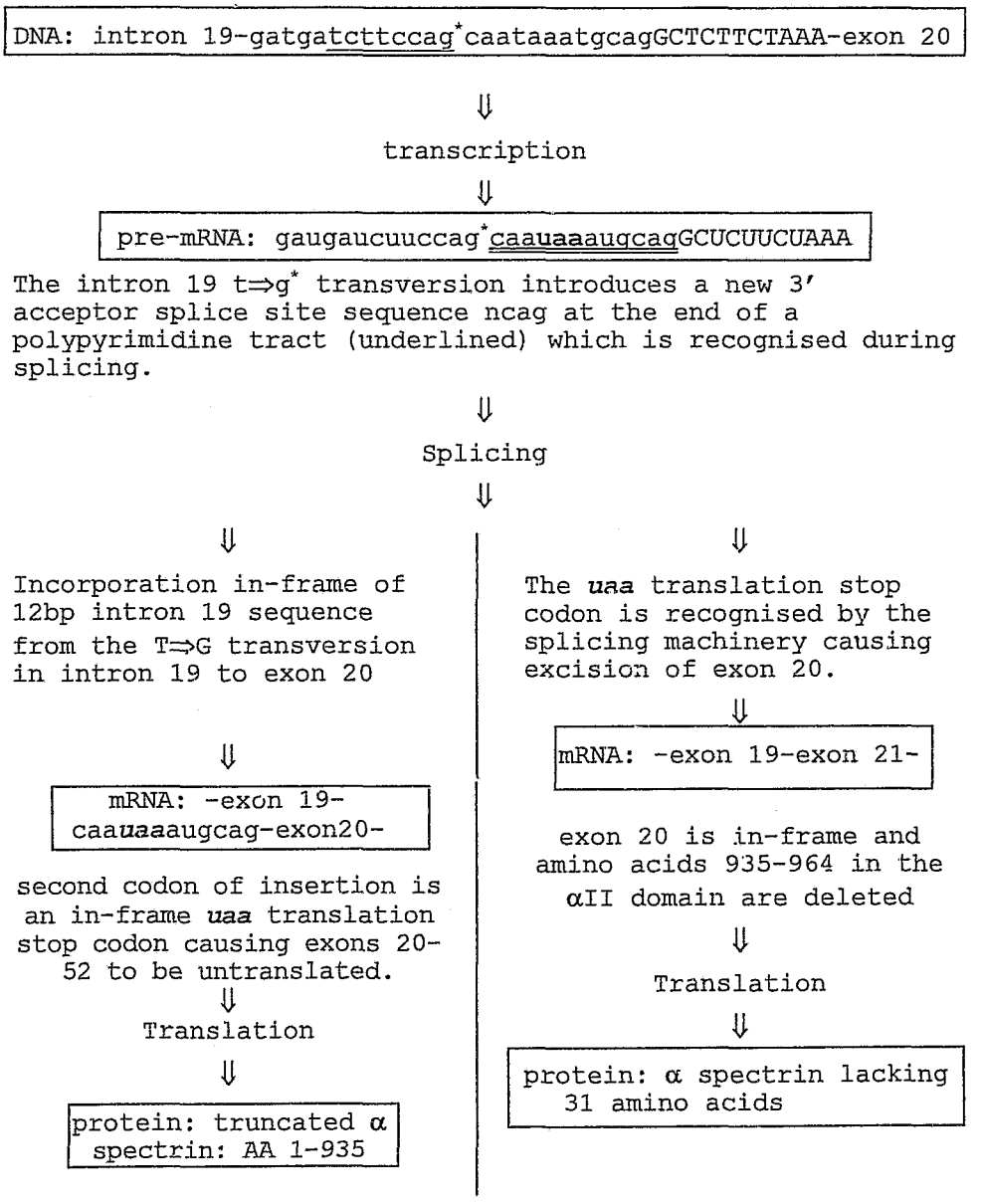


Figure 24.

Proposed mechanism for the splicing of Spectrin Johannesburg RNA transcripts and translation of the resulting mRNA. Spectrin Johannesburg is characterised by a T⇒G transversion in intron 19, 13bp upstream from exon 20 (marked with an asterisk). The

sequence of α spectrin intron 19 is marked by lower case letters and exon 20 sequence is in capital letters.

the first event shown in figure 24, 12bp of intron 19 sequence from the T⇒G mutation to the start of exon 20 are retained in-frame with exon 20. Thus the insertion sequence matched the intron 19 sequence exactly. In the second splicing event, codon two of the 12bp insertion, a UAA translation stop codon, is recognised by the splicing machinery which excises exon 20 by an unknown mechanism. Both splicing events occur resulting in partial skipping of exon 20. The mother was heterozygous for the intron 19 mutation and produced the normal as well as both abnormal mRNA species demonstrating that the single mutation was adequate to cause both splicing events.

The excision of an exon in a pre-mRNA transcript due to the presence of a premature nonsense codon is an unusual event that has been documented in the human fibrillin and ornithine δ-aminotransferase gene transcripts (Dietz et al, 1993). The two abnormal αII domain RT-PCR products from the probands were consistently of similar relative intensities on agarose gels. This implied that the amounts of each mRNA template were similar and therefore there was no bias for the splicing or degradation of one abnormal message over the other. This is in contrast to spectrin St Claude (Fournier et al, 1997) where an increased level of the exon 20 skipped message was observed.

Investigations using yeast mRNA have shown that premature stop codons within the first two-thirds to three-quarters of the coding sequence of an mRNA template target the molecule for early destruction only in the presence of a specific downstream sequence (Jacobson and Peltz, 1996). α Spectrin does not contain this downstream

sequence (5'- TGCTGATGCTTTCTCTGCTGATGC-3') which is consistent with the normal stability observed for the α spectrin mRNA containing the premature stop codon.

In the homozygous probands, processing of the transcripts of the mutant allele is divided equally into the two abnormal species. The same would be expected to occur in the heterozygous mother. On agarose gel separations of the mother αII domain RT-PCR products, the intensity of the smaller band, amplicons of the exon 20 skipped mRNA, was lower than the intensity of the larger band which containing both the normal mRNA and message with the 12bp insertion. Assuming an equal division of the two abnormal messages in the mother, the intensity of the smaller band therefore reflected the amount of mRNA carrying the 12bp insertion. In the absence of factors targeting the abnormal RNA messages for premature degradation, the mother's population of α spectrin mRNA is predicted to be divided into normal, exon skipped and 12bp inserted messages in a ratio of approximately 2 : 1 : 1.

Translation of the RNA message carrying the 12bp intron 19 insertion, figure 24, is stopped at codon 935 by the in-frame UAA stop codon introduced by the 12bp insertion after the addition of a glutamine residue. This molecule lacks the C-terminal 1495 amino acids (Winkelmann et al, 1990). Spectrin heterodimer formation is directed by the association of complementary essential nucleation sites in the C-terminal repeats 20 and 21 of α spectrin and repeats 1 and 2 of β spectrin (Speicher et al , 1992). The truncated α spectrin does not have the nucieation sites and therefore

cannot form heterodimers with β spectrin. The integral protein band 3 provides the high affinity attachment to the erythrocyte membrane for spectrin via ankyrin onto β spectrin (Bennett and Stenbuck, 1979a and b). Therefore the truncated α spectrin remains monomeric and unable to attach to the membrane. Cytosolic spectrin monomers are degraded at all stages of erythropoiesis (Hanspal and Palek, 1987). In the probands and the mother the unincorporated truncated α spectrin is predicted to be proteolysed during erythrocyte membrane biogenesis (Hanspal and Palek, 1987) which explains its absence in membrane studies (Zail and Coetzer, 1984).

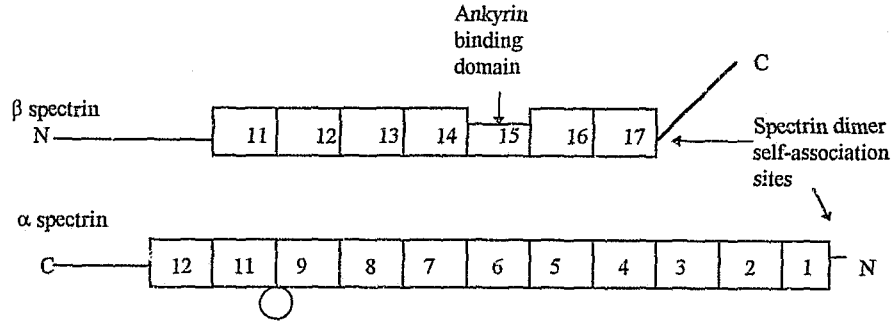
The primary structure of α spectrin (Sahr et al, 1990) is divided into a series of 106 homologous amino acid repeat sequences which form triple helical bundles (Speicher and Marchesi, 1984 and Yan et al, 1993). Translation of the exon 20 skipped message which deletes amino acids 935-965 is predicted to remove the B helix of α spectrin repeat 9 in the αII domain. The αII domain tryptic peptides are derived from a common tryptic cleavage site at amino acid 680 (DiPaolo et al, 1993) and secondary sites at amino acids 1046, 981, 939 and 916 that give rise to the 46kDa, 35kDa, 30kDa and 25kDa peptides respectively which are described in figure 19. The probands' α spectrin is derived from translation of exon 20 skipped message and thus amino acid 939 is deleted which explains the absence of the 30kDa peptide in tryptic digests. The conformation around the tryptic cleavage site at amino acid 981, inside the SH3 domain of atypical repeat 10 (Sahr et al, 1990), is presumably disrupted by the deletion of amino acids 935-965 in the probands as evidenced by the absence of

the 35kDa αII peptide from spectrin digests. The 25kDa peptide was unaffected in the probands because the tryptic cleavage site at amino acid 916 is too far away to be affected by the deletion.

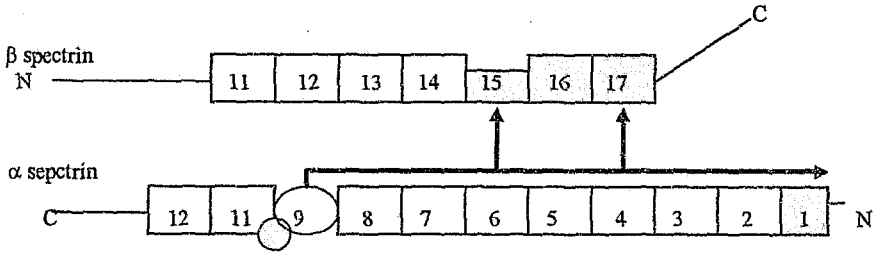
hSp assays identified the probands' α spectrin chain as the cause of the previously described functional defects of the probands' spectrin (Coetzer and Zail, 1992 and Zail and Coetzer, 1984). The only structural change found was in the probands' spectrin αII domain and thus the underlying cause for the probands' reduced spectrin dimer self-association, defective spectrin-ankyrin binding and decreased membrane spectrin content was an α spectrin chain lacking the B helix of repeat α9 in the repeat conforamtional unit of spectrin (figure 3). This was further confirmed by the absence of mutations in the genomic DNA coding for the relevant functional domains. αII domain mutations have been reported that cause mild dimer self-association defects. In spectrin Oran (Alloisio et al, 1993) and Jendouba (Alloisio et al, 1992) helices A and B of repeat α8 respectively are deleted resulting in mild dimer self-association defects. The deletion of the B helix in repeat α9 of Spectrin Johannesburg is consistent with these findings as it causes mildly reduced dimer self-association. The second more profound effect of Spectrin Johannesburg is the marked reduction in the ability of the probands' spectrin to bind ankyrin. This is a novel finding and implies that the abnormal αII domain exerts a long range influence on the ankyrin binding domain of the neighbouring β spectrin in the heterodimer.

In figure 25 adjacent portions of the α and β spectrin chains in the vicinity of the β

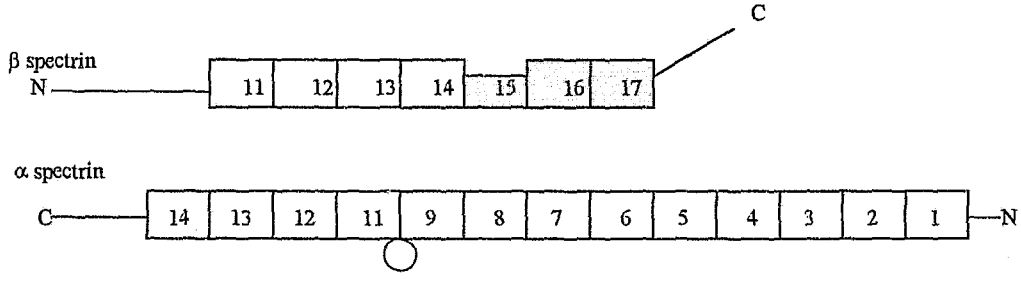
(A)


Ankyrin
binding
domain
C
β spectrin
N
11
12
13
14
15
16
17
Spectrin dimer
self-association
sites
α spectrin
C
12
11
9
8
7
6
5
4
3
2
1
N

(B)


C
β spectrin
N
11
12
13
14
15
16
17
α sepctrin
C
12
11
9
8
7
6
5
4
3
2
1
N

(C)


C
β spectrin
N
11
12
13
14
15
16
17
α spectrin
C
14
13
12
11
9
8
7
6
5
4
3
2
1
N

(D)

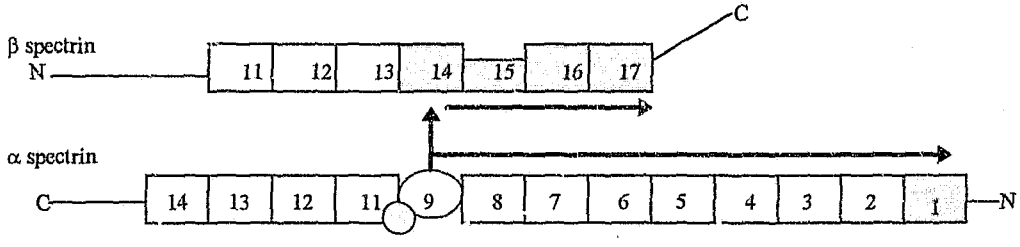

C
β spectrin
N
11
12
13
14
15
16
17
α spectrin
C
14
13
12
11
9
8
7
6
5
4
3
2
1
N

Figure 25.

Diagrammatic representation of possible registers of the spectrin triple helical bundles α1-14 with β spectrin β11-17 and possible mechanisms for the disruption of β spectrin functions by Spectrin Johannesburg. Spectrin triple helical bundle conformational units are shown as blocks, the α spectrin SH3 domain is drawn as a circle, the mutant α9 lacking the B helix from Spectrin Johannesburg is represented by an ellipse and structures with abnormal conformation are filled. Two possible registers of triple helical bundles are shown in (A) and (C). In (B) the abnormal α9 triple helical bundle of Spectrin Johannesburg, opposite β12, is too far from the ankyrin binding and dimer self-association sites of β spectrin to cause direct disruption. In this model, a structural distortion form the abnormal α9 is transmitted along the α chain (arrows) to points opposite the functional domains of β spectrin in β15-17. In (D) the altered α9 conformation, opposite β14, is close enough to directly disrupt β14-17 thus affecting β spectrin function directly. Models (B) and (D) do not exclude the possibility that disruptive signals are propagated along α spectrin to the N-terminal α1 domain to cause self-association defects in this domain as well.

spectrin ankyrin binding domain are shown. The ankyrin binding domain is situated on β spectrin between homologous repeats 15 and 16. In recombinant peptide studies this domain was shown to fold independently (Kennedy et al, 1991) and it therefore does not require α spectrin interactions within the heterodimer to fold. Thus within a dimer, the probands' mutant α spectrin is a disruptive influence on the β spectrin ankyrin binding domain. The ankyrin binding site is adjacent to the β spectrin dimer self-association site in repeat 17, figure 25, and it is therefore possible that these two functional domains are affected by a single disruptive mechanism. To model this disruption and link it to the altered αII domain structure of Spectrin Johannesburg it is necessary to consider the associations between the α and β spectrin subunits in a heterodimer.

The extended nature of spectrin and the specificity of the nucleation sites that direct α and β spectrin heterodimer formation (Speicher et al, 1992) imply a specific alignment of the homologous repeats along the length of the molecule. This register of the repeat units is not known but can be approximated by assuming each one has a similar length. Simple models based upon this premise place α spectrin repeat 9 opposite β spectrin repeats 14 (Speicher et al, 1992) and 12 (DeSilva et al, 1997) as shown in figure 25. After tryptic digestion of spectrin, non-covalent side-by-side interactions between the monomers are maintained (Speicher et al, 1992) implying close specific associations of the monomers along their lengths. These interactions include: an association of the 80kDa αI domain with a β spectrin peptide incorporating the βI and βII domains that

include the ankyrin binding domain (Morrow et al, 1980) and a strong association of the αII domain with digested spectrin dimers (Speicher et al, 1992). Thus within the region of the ankyrin binding and dimer self-association domains there are extensive α and β subunit interactions. There is also evidence that the oligomeric state of spectrin influences its spectrin-ankyrin binding affinity (Cianci et al, 1988) implying communication between the triple helical conformational units.

Given the extensive associations between subunits in the spectrin heterodimer, it is possible to postulate mechanisms whereby the abnormal αII domain of Spectrin Johannesburg disrupts ankyrin binding and self-association. The disruptions would have to be caused by long range interactions between the altered αII domain and the relevant β-spectrin domains. The disruption emanates from the altered conformation of the α9 triple helical bundle which, for this model, perturbs the normal fit between subunits as a result of the removal of helix B in repeat α9. Figure 25 describes two possible mechanisms for the long range interactions between the altered α9 conformation and the functional domains affected. In the case where the altered α9 triple helical bundle is distant from the ankyrin binding site in β15 and β16, as in the case where it is placed opposite β12, the disruptive influence of the altered α9 unit is propagated along the αspectrin chain to points adjacent to β15-17 as shown in figure 25 (C). This then detrimentally affects the β spectrin functions. Where the altered α9 and β14 triple helical bundles are opposite one another, the mutant α9 is spatially

close enough to the ankyrin binding domain to directly influence the β spectrin-ankyrin binding site and with it the neighbouring β17 dimer self-association domains as shown in figure 25 (D). Alternatively, the perturbation in the probands' spectrin dimer self-association may be due to a propagation of a disruptive influence along the α spectrin to the α1 triple helical bundle. This possibility is not mutually exclusive with a model suggesting a disrupted β17 triple helical bundle.

A full explanation of the spatial and mechanistic relationships between the altered αII domain structure of Spectrin Johannesburg and the functional domains over which it exerts its influence requires an understanding of two particular aspects of spectrin structure and function. These are the joins between repeating spectrin triple helical conformational segments and the interactions between monomers in a heterodimer. These properties are not well understood and a complete mechanistic explanation of the Spectrin Johannesburg phenotype awaits a better understanding of these aspects.

Figure 26 summarises the phenotypic expression of individuals homozygous for the normal α spectrin allele and heterozygotes and homozygotes for the Spectrin Johannesburg genotype. A heterozygote, figure 26(B), for Spectrin Johannesburg co-expresses the two abnormal Spectrin Johannesburg α spectrin chains with normal monomer in a predicted ratio of 2 : 1 : 1. The truncated Spectrin Johannesburg α subunit cannot from heterodimers and is degraded. The second α spectrin, derived from the exon 20 skipped message, can form heterodimers but has a severe ankyrin binding defect and

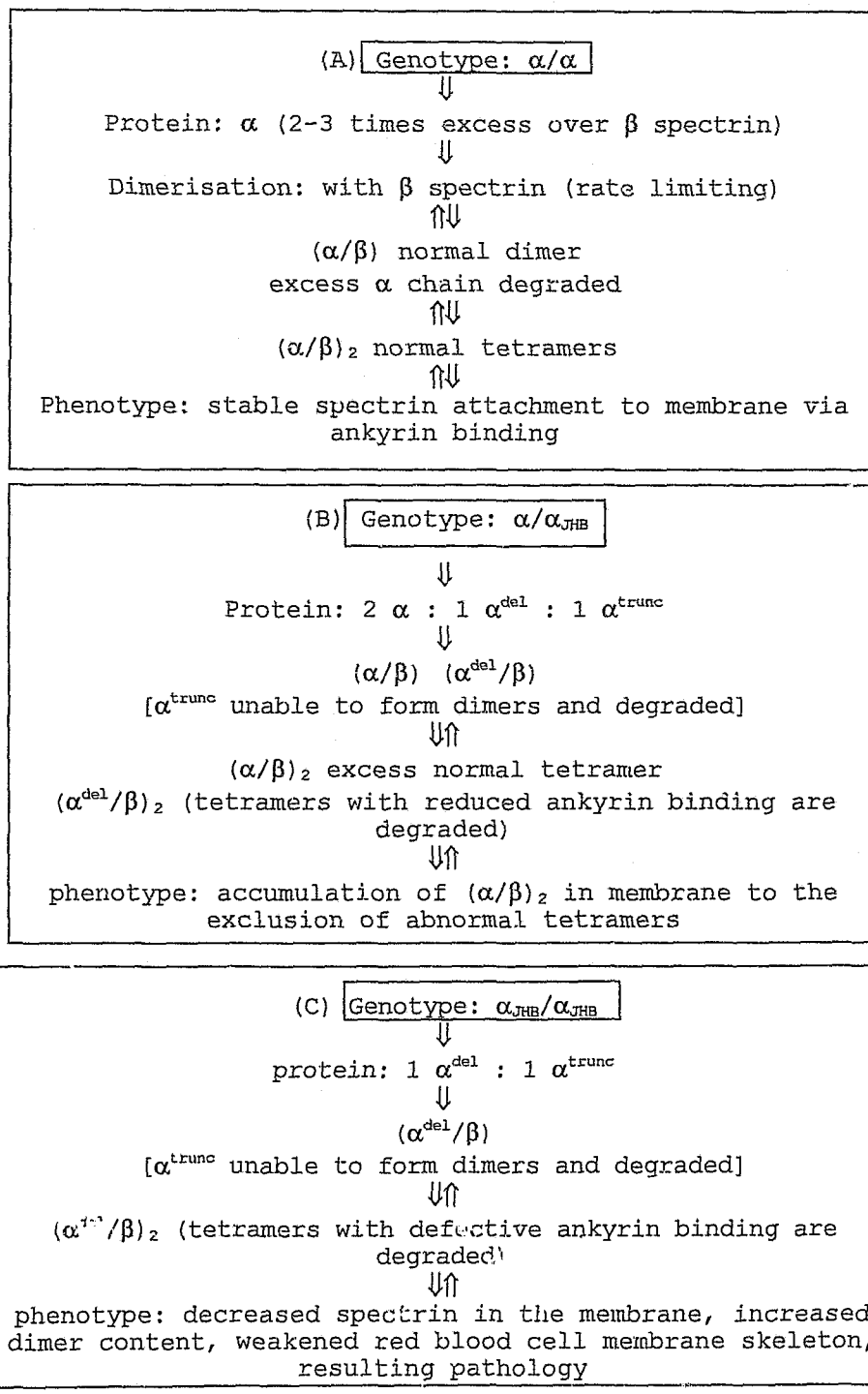

(A) Genotype: α/α
Protein: α (2-3 times excess over β spectrin)
Dimerisation: with β spectrin (rate limiting)
(α/β) normal dimer
excess α chain degraded
(α/β)2 normal tetramers
Phenotype: stable spectrin attachment to membrane via ankyrin binding
(B) Genotype: α/αJHB
Protein: 2 α : 1 αdel : 1 αtrunc
(α/β) (αdel/β)
[αtrunc unable to form dimers and degraded]
(α/β)2 excess normal tetramer
(αdel/β)2 (tetramers with reduced ankyrin binding are degraded)
phenotype: accumulation of (α/β)2 in membrane to the exclusion of abnormal tetramers
(C) Genotype: αJHB/αJHB
protein: 1 αdel : 1 αtrunc
(αdel/β)
[αtrunc unable to form dimers and degraded]
(αdel/β)2 (tetramers with defective ankyrin binding are degraded)
phenotype: decreased spectrin in the membrane, increased dimer content, weakened red blood cell membrane skeleton, resulting pathology

Figure 26

Summary of the phenotypic effects of heterozygosity and homozygosity for the Spectrin Johannesburg (Sp_{JHB}) genotype. In the diagram, α represents the normal α spectrin allele and gene product. α_{JHB} represents the Spectrin Johannesburg allele and α^{del} represents the Spectrin Johannesburg gene product lacking the amino acids coded for by exon 20 and α^{trunc} is the truncated Spectrin Johannesburg gene product.

reduced dimer self-association. Heterodimers containing this defective α spectrin cannot compete with heterodimers carrying the normal α spectrin allele which is in a 2:1 excess. Despite a reduction in the amount of normal α spectrin chains the overexpression of α monomers (Hanspal and Palek, 1987) during red cell membrane biogenesis results in normal levels of spectrin incorporation into the membrane. Thus normal α spectrin at normal levels is expressed on the membrane. This explanation is consistent with the normal status observed for the heterozygous mother's α spectrin chain and membrane spectrin content. In the homozygote for Spectrin Johannesburg, figure 26(C), half the α spectrin chains are truncated and half have the exon 20 deletion. Only the exon 20 deleted α spectrin can be incorporated into heterodimers that then have ankyrin binding and dimer self-association defects. Although the amount of available α spectrin is effectively halved during erythrocyte membrane biogenesis, the deficiency should be offset by the overexpression of α chains. However the severe ankyrin binding defect induced by the mutant α spectrin chain results in lowered spectrin incorporation into the membrane and 20% spectrin deficiency. Thus, as observed, the homozygous probands both expressed a single defective α spectrin with abnormal tetramer formation and ankyrin binding.

The experimental data from this work shows that the lowered ankyrin binding of the probands' spectrin was the cause of the erythrocyte spectrin deficiency; an uncommon finding in HE (Palek and Jarolim, 1995). These conclusions differ from the report of

spectrin St Claude where the spectrin deficiency was attributed to a low expression α spectrin allele (Fournier et al, 1997).

Peripheral blood smears from the probands' showed predominantly elliptocytes with some microspherocytes and poikilocytes. These altered morphologies are due to the inability of the erythrocytes to withstand the stresses of circulation. In the circulation erythrocytes withstand tremendous forces (Palek, 1986). In the probands, the protein skeleton attached to the erythrocyte membrane has reduced spectrin content caused by the severe, vertical (Palek, 1986), spectrin-ankyrin binding defect. This weakens the membrane which cannot withstand forces that result in some loss of lipid and microspherocyte formation. Spectrin dimer self-association defects are horizontal weaknesses. In severe cases these result in elliptocytic deformation of red cells and HE (Palek and Jarolim, 1995b). The probands' dimer self-association defect is mild and alone would be predicted to have resulted in some permanent erythrocyte deformation (Lawler et al, 1985). However the spectrin depletion of the membrane skeleton combined is combined with this mild defect. The weakened erythrocytes are removed prematurely from circulation in the spleen (Chen and Weiss, 1973) which causes the observed anaemia.

6. Conclusion

This investigation identified an α spectrin intron 19 T⇒G transversion 13 bp upstream in the α spectrin exon 20 boundary which was named Spectrin Johannesburg. This mutation creates a novel pre-mRNA splice site which is recognised to the exclusion of the normal site. Two abnormal RNA messages are produced: one with a 12bp in-frame insertion that includes an in-frame translation stop codon and the second with an in-frame deletion of exon 20. The second message is produced by skipping of exon 20 after recognition of the in-frame stop codon after the novel splice site. The phenomenon of partial exon skipping due to an in-frame stop codon within an exon is rarely observed. Thus this work sheds light on the nature of splicing mechanisms.

Two defective α spectrin species result from translation of the abnormal Spectrin Johannesburg RNA messages. The first is a molecule that cannot form heterodimers which is not incorporated into the membrane. The second message produces an α spectrin with an altered αII domain. The α spectrin carrying the altered αII domain forms heterodimers and is incorporated into the membrane. The altered conformation of the mutant α spectrin severely disrupts the ankyrin binding of the neighbouring β spectrin in the heterodimer and causes a mild spectrin dimer self-association defect. The Spectrin Johannesburg mutation was found in two probands from a white South African kindred who suffer from a severe atypical form of HE. The HE is ultimately caused by the functional defects attributable to the defective αII domain of the α

spectrin expressed on their erythrocyte membranes. This therefore demonstrates an abnormal and pathological interaction between the α and β spectrin chains in the heterodimer. This work thus provides insights into the nature of hereditary anaemia, the structure and functions of spectrin and the complex relationship between genotype and phenotype.

The kindred studied is of Afrikaans origin and their parents apparently unrelated. Since the probands are homozygous for the Spectrin Johannesburg allele the parents are obligate heterozygotes. Future studies will include screening unrelated white and black South African control subjects to measure the prevalence of the Spectrin Johannesburg allele. In an initial study of white subjects, two mutant alleles out of 134 were detected (T. Coetzer and D. Van Zyl private communication).

7. References

Alloisio, N., Wilmotte, R., Marechal, J., Texier, P., Denoroy, L., Feo, C., Benhadji-Zouaoui, Z. and Delaunay, J. (1993) A splice site mutation of α-spectrin gene causing skipping of exon 18 in hereditary elliptocytosis. *Blood* **81**, 2791-2798.

Alloisio, N., Wilmotte, R., Morle, L., Baklouti, F., Marechal, J., Ducluzeau, M.-T., Denoroy, L., Feo, C., Forget, B. G., Kastally, R. And Delaunay, J. (1992) Spectrin Jendouba: An $\alpha^{II/31}$ spectrin variant that is associated with elliptocytosis and carries a mutation distant from the dimer self-association site. *Blood* **80**, 809-815.

Amin, K. M., Scarpa, A. L., Winkelmann, J. C., Curtis, P.J. and Forget, B. (1993) The exon-intron organisation of the human erythroid β-spectrin gene *Genomics* **18**, 118-125.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.M., Seidman, J.G., Smith, J.A., Struhl, K.M. Current protocols in molecular biology. Wiley Interscience.

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

Becker, P.S., Schwartz, M.A., Morrow, J.S. and Lux, S.E. (1990) Radiolabel-transfer cross-linking demonstrates that protein 4.1 binds to the N-terminal region of β spectrin and to actin in binary interactions. *Eur. J. Biochem.* **193**, 827- 836.

Bennett, V. (1985) The membrane skeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* 54, 273-304.

Bennett, V. (1989) . The spectrin-actin junction of erythrocyte membrane skeletons. *Biochem Biophys Acta* **988**, 107-121.

Bennett, V. and Branton, D. (1977) Selective association of spectrin with the cytoplasmic surface of human erythrocyte plasma membranes. *J. Biol. Chem.* **252**, 2753-2763.

Bennett, V. and Stenbuck, P. J. (1979a) Identification and partial purification of ankyrin, the high affinity membrane attachment site of human erythrocyte spectrin. *J. Biol. Chem.* **254**, 2533-2541.

Bennett, V. and Stenbuck, P. J. (1979b) The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. *Nature* **280**, 468-473.

Blanco, F.J., Oritz, A.R. and Serrano, L. (1997) ^{1}H and ^{15}N NMR assignment and solution structure of the SH3 domain of spectrin: comparison of unrefined and refined structure sets with the crystal structure. *J. Biolmolecular NMR* **9**, 347-357.

Bolton, A.E. and Hunter, W.M. (1973) The labelling of proteins to high radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.*, **133**, 529-539.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Buday, L. and Downward, J. (1993) Epidermal growth factor regulates the p21ras through the formation of a complex of receptor, Grb2 adapter protein and Sos nucleotide exchanger. *Cell* **73**, 611-620.

Burke, J., Zail, S.S. and Coetzer, T.L. (1997c) Spectrin Johannesburg: An abnormal spectrin αII domain due to partial exon skipping decreases spectrin self-association and ankyrin binding resulting in hereditary elliptocytosis. *Blood* **90** Suppl 1, abstr 3-I.

Burke, J.P., Zail, S.S. and Coetzer, T.L. (1997b) Spectrin Johannesburg: A novel α spectrin defect. Proceedings of the South African Society of Biochemistry and Molecular Biology, 14th conference, Grahamstown, p 89.

Burke, J.P., Zail, S.S. and Coetzer, T.L. (January 1997a) Exon skiping caused by a stop codon. Proceedings of the South African Society of Biochemistry and Molecular Biology, 14th conference, Grahamstown, p 46.

Byers, T.J. and Branton, D. (1985) Visualisation of the protein associations in the erythrocyte membrane skeleton. *Proc. Natl. Acad. Sci. USA* **82**, 6153-6157.

Byers, T.J., Brandin, E., Lue, R.A., Winograd, E. and Branton, D. (1992) The complete sequence of *Drosophila* β-spectrin reveals supra-motifs comprising eight 106 amino acid sequences. *Proc. Natl. Acad. Sci. USA* 89, 6187-6191.

Calvert, R., Bennett, V. and Gratzer, W. (1980a) Properties and structural role of the subunits of human spectrin. *Eur. J. Biochem.* **107**, 355-361.

Calvert, R., Ungewickell, E. and Gratzer, W. (1980b) A conformational study of spectrin. *Eur. J. Biochem.* **107**, 363-367.

Carraway, K.L. and Carraway, C.A.C. (1989) Membrane-cytoskeleton interactions in animal cells. *Biochem. Biophys. Acta* **988**, 147-171.

Chen, L.T. and Weiss, S. (1973) The role of the sinus wall in the passage of erythrocytes through the spleen. *Blood* **41**, 529-537

Chomczynski, P. and Sacchi, N. (1978) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Cianci, C. D., Giorgi, M. and Morrow, J. S. (1988) Phosphorylation of ankyrin down-regulates its cooperative interaction with spectrin and protein 3. *J. Cell. Biol.* **37**, 301-315.

Clarke, M. (1971) Isolation and characterisation of a water soluble protein from bovine erythrocyte membranes. *Biochem. Biophys. Res. Commun.* **45**, 1063-1070.

Coetzer, T.L. and Palek J. (1986) Partial spectrin deficiency in hereditary pyropoikilocytosis. Blood **67**, 919-924.

Coetzer, T.L. and Zail, S. S. (1981) Trytpic digestion of spectrin in variants of hereditary elliptocytosis. *J. Clin. Invest* **67**, 1241-1248.

Coetzer, T.L. and Zail, S.S. (1982) Spectrin tetramer dimer-tetramer equiiibrium in hereditary elliptocytosis. *Blood* **59**, 900-905.

Coetzer, T.L., Beeton, L., Van Zyl, D., Field, S.P., Agherdien, A., Smart, E. and Damele, G.L. (1996) South East Asian ovalocytosis in a South African kindred with haemolytic anaemia. *Blood* **87**, 1656-1657.

Coetzer, T.L., Lawler, J. and Palek, J. (1985) A new structural variant of the αIII domain of red blood cell spectrin. Blood **70**, abstract 30a.

Coetzer, T.L., Lawler, J., Prchal, J.T. and Palek, J. (1987) Molecular determinants of clinical expression of hereditary elliptocytosis and pyropoikilocytosis. *Blood* **70**, 766-772.

Cohen, C., M. and Foley, S.F. (1984) Biochemical characterization of complex formation by human erythrocyte spectrin, protein 4.1 and actin. *Biochemistry* **32**, 6091-6098.

Cohen, C. M. and Langley, R. C. (1984) Functional Characterization of human erythrocyte spectrin α and β chains: Association with actin and erythrocyte protein 4.1. *Biochemistry*, **23**, 4488-4495.

Conboy, J.G. (1993) Structure, function, and molecular genetics of erythroid membrane skeletal protein 4.1 in normal and abnormal red blood cells. *Semin. Hematol.* **30**, 58-73.

Conboy, J.G., Chan, J., Chasis J.A., Kan, Y.W. and Mohandas, N. (1991) Tissue- and development-specific alternative RNA splicing regulates expression of multiple isoforms of erythroid membrane protein 4.1. *J. Biol. Chem.* **266**, 8273-8280.

Correas, I, Leto, T.L. and Speicher, D.W. (1986) Identification of the functional site of erythrocyte protein 4.1 involved in spectrin-actin associations. *J. Biol. Chem.* **261**, 3310-3315.

Corsi, D., Galluzzi, L., LeComte, M.C. and Magnani, M. (1997) Identification of alpha-spectrin domains susceptible to ubiquitination. *J. Biol. Chem.* **272**, 2977-2983.

Dean, A. D., Greenwald, J .E. (1995) Use of Filtered Pipet Tips to Elute DNA from Agarose Gels. *Biotechniques* **18**, 980.

Delaunay, J. and Dhermy, D. (1993) Mutations involving the spectrin heterodimer contact site: clinical expression and alterations in specific function. *Semin. Hematol.* **30**, 21-33.

DeSilva, T. M., Peng, K., Speicher, K. D. and Speicher, D. W. (1992) Analysis of human red cell spectrin tetramer (head-to-head) assembly using complementary univlent peptides. *Biochemistry*, **31**, 10872-10878.

DeSilva, T.M., Harper, S.L., Kotula, L., Hensley, P., Curtis, P.J., Otvos, L. Jr. and Speicher, D.W. (1997) Physical properties of a single-motif erythrocyte spectrin peptide: A highly stable independently folding unit. Biochem. **36**, 3991-3997.

Dietz, H. C., Valle, D., Francomano, C. A., Kendzior Jr, R. J., Pyeritz, R. E. and Cutting, C. (1993) The Skipping of Constitutive Exons *in vivo* Induced by Nonsense Mutations. *Science* **259**, 680-682.

DiPaolo, B. R., Speicher, K. D., and Speicher, D. W. (1993) Identification of the Amino Acid Mutations Associated With Human Erythrocyte Spectrin αII Domain Polymorphims. *Blood* **82**, 284-291

Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**, 119-130.

Fairbanks, G., Steck, T.L., Wallach, D.F.H. (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**, 2606-2617.

Feller, S.M., Ren ,R., Hanafusa, H. And Baltimore, D. (1994) *Trends in Biochemistry* **19**, 453-458.

Fournier, C.M., Nicolas, G., Gallagher, P. G., Dhermy, D., Grandchamp, B., LeComte, M. C. (1997) Spectrin St Claude, a Splicing Mutation of the Human α-Spectrin Gene Associated With Severe Poikilocytic Anaemia. *Blood*, **89**, 4584-4590.

Fowler, V. M. (1990) Tropomodulin: A cytoskeletal protein that binds to the end of erythrocyte tropomyosin and inhibits tropomyosin binding to actin. *J. Cell. Biol.* **111**, 471-482.

Fukushima, Y., Byers, M.G. and Watkins, P,C. (1990) Assignment of the gene for β-spectrin (SPTB) to chromosome 14q23->q24.2 by *in situ* hybridisation. *Cytogenet Cell Genet* **53**, 232-233.

Gallagher, P. G. and Forget, B.G. (1996) Hematologically important mutations: Spectrin variants in hereditary elliptocytosis and hereditary pyropoikilocytosis. *Blood cells, molecules and diseases* **22**, 254-258.

Gallagher, P.G. and Forget, B.G. (1993) Spectrin genes in health and disease. *Semin. Hematol.* **30**, 4-21.

Gallagher, P.G., Tse, W.T., Mohandas, N., Marchesi, S.L. and Forget, B.G. (1992) Spectrin Providence: A defect of beta spectrin ($\beta^{2109\ ser\text{-}pro}$) homozygosity for which is associated with fatal hydrops fetalis. *Blood* **80**, 145a(abstr)

Gardner, K. and Bennett, V. (1987) Modulation of spectrin-actin assembly by erythrocyte adducin. *Nature*, **328**, 359-362.

Gilligan, D.M. and Bennett, V. (1993) The junctional complex of the membrane skeleton. *Semin. Hematol.* **30**, 74-83.

Gomperts, E. D., Cayanis, F., Metz, J. and Zail, S.S. (1973) A red cell protein abnormality in hereditary elliptocytosis. *Br. J. Haematol.* **25**, 415-420.

Goodman, S. R. and Weidner S. A. (1980) Binding of α_2-β_2 Spectrin to Human Erythrocyte Membranes *J. Biol. Chem.* **255**, 8082-8086.

Hadley, T., Saul, A., Lamont, G., Hudson, D.E., Miller, L.H. and Kidson, C. (1983) Resistance of melanesian elliptocytes (ovalocytes) to invasion by *Plasmodium knowlesi* and *Plasmodium falciparum* malaria pararsites *in vitro*. *J. Clin. Invest.* **71**, 780-782.

Hanspal, M. and Palek, J. (1987) Synthesis and assembly of membrane skeletal proteins in mammalian red cell precursors. *J. Cell. Biol.* **105**, 1417-1424.

Hanspal, M., and Palek, J. (1992) Biogenesis of normal and abnormal red blood cell membrane skeleton. *Semin. Hematol.* **29**, 205-319.

Hanspal, M., Hanspal, J., Kalraiya, R., Liu, S.C., Sahr, K.E., Howard, D., and Palek, J. (1992a) Asynchronous synthesis of membrane skeletal proteins during terminal maturation of murine erythroblasts. *Blood* **80**, 530-539.

Hanspal, M., Hanspal, J., Kalrayia, R. and Palek, J. (1992b) Expression and synthesis of the band 3 protein initiates the formation of a stable membrane skeleton in murine Rauscher transformed erythroid cells. *Eur. J. Cell Biol.* **58**, 313-318.

Harris, A.S. and Morrow, J.S. (1990) Calmodulin and calcium-dependent protease I coordinately regulate the interaction of fodrin with actin. *Proc. Natl. Acad. Sci. USA* **87**, 3009-3013.

Harris, H.W., Jr., and Lux, S.E. (1980) Structural characterisation of the phosphorylation sites of human erythrocyte spectrin. *J. Biol. Chem.* **255**, 11512-11520.

Harris, W.H.Jr., Levin, N. And Lux, S.E. (1980) Comparison of the phosphorylation of human erythrocyte spectrin in the intact red cell and in various cell-free systems. *J. Biol. Chem.* **255**, 11521-11525.

Hu, R.-J. and Bennett, V. (1991) *In vitro* proteolysis of brain spectrin by calpain I inhibits association of spectrin with ankyrin-independent membrane binding site(s). *J. Biol. Chem.* **266**, 18200-18205.

Huebner, K., Palumbo, A. P., Isobe, M., Kozac, C. A., Monaco, S., Rovera, G., Croce, C. M. and Curtis, P. J. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3790-3793.

Husain-Chisti, A., Faquin, W., Wu, C.C. and Branton, D. (1989) Purification of erythrocyte dematin (protein 4.9) reveals an endogenous protein kinase that modulates actin-bundling activity. *J. Biol. Chem.* **264**, 8985-8991.

Husain-Chisti, A., Levin, A. and Branton, D. (1988) Abolition of actin-bundling by phosphorylation of human erythrocyte protein 4.9. *Nature* **334**, 718-721.

Inaba, M., Guptka, K.C., Kuwabara, M., Deamidation of human erythrocyte protein 4.1: possible roles in aging. (1992). *Blood* **79**, 3355-3361.

Jacobson, A. and Peltz, S., W. (1996) Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. *Annu. Rev. Biochem.* **65**, 693-739.

Jarolim, P., Palek, J., Amato, D. (1991) Deletion in erythrocyte band 3 gene in malarial resistant South East Asian Ovalocytosis. *Proc. Natl. Acad. Sci. USA* **88**, 11022-11026.

Kennedy, S. P., Warren, S. L., Forget, B. G. and Morrow, J. S. (1991) Ankyrin Binds to the 15th Repetitive Unit of Erythroid and Non-erythroid β-Spectrin *J. Cell. Biol.* **115**, 267-277.

Kennedy, S.P., Weed, S.A., Forget, B.G. and Morrow, J.S. (1994) A partial structural repeat forms the heterodimer self-association site of all β-spectrins. *J. Biol. Chem.* **269**, 11400-11408.

Key, J.A. (1921) Studies on erythrocytes, with special reference to reticulum. *Arch Intern Med.* 28, 511. (From Sorette et al Blood 1992 **80**(1) 249-254).

Khodadad, J.K., Waugh, R.E., Podolski, J.L., Josephs, R. and Steck, T.L. (1996) Remodeling the shape of the skeleton in the intact red cell. *Biophys. J.* **70**, 1036-1044.

Knowles, W. J. and Bologna, M. L. (1983) Isolation of the chemical domains of human erythrocyte spectrin. *Methods in Enzymol.* **73**, 307-313.

Knowles, W.J., Bologna, M. L., Chasis, J.A., Marchesi, S.L. and Marchesi, V.T. (1984) Common structural polymorphisms in human erythrocyte spectrin *J. Clin. Invest.* **73**, 973-979.

Kotula, L., DeSilva, T. M., Speicher, D. W. and Curtis, P. J. (1993) Functional characterisation of recombinant human red cell α-spectrin polypeptides containing the tetramer binding site. *J. Biol. Chem.* **268**, 14788-14793.

Kotula, L., Laury-Kleintop, L.D., Showe, L., Sahr, K., Linnenbach, A.J., Forget, B and Curtis, P.J. (1991) The exon-intron organisation of the human erythrocyte α-spectrin gene *Genomics* **9**, 131-140.

Koury, M.J., Bondurant, M.C. and Rana, S.S. (1987) Changes in erythroid membrane proteins during erythropoietin-mediated terminal differentiation. *J. Cell. Physiol.* **133**, 438-448.

LaBrake, C.,C., Wang, L., Keiderling, T.A. and Fung, L.W. (1993). Fourier transform infrared spectroscopic studies of the secondary structure of spectrin under different ionic strengths. *Biochemistry* **32**, 10296-10302.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (Lond.) **227**, 680-685.

Lambert, S. Yu, H., Prchal, J.T., Ruff, P., Speicher, D., Cheung, M.C., Kan, Y.W. and Palek, J. (1990) cDNA sequence for human erythrocyte ankyrin. *Proc. Natl. Acad. Sci. USA* **87**, 1730-1734.

Lawler, J., Coetzer, T. L., Palek, J., Jacob, H.S. and Luban, N. (1985) Spα$^{I/65}$: A new variant of the α subunit of spectrin in hereditary elliptocytosis. *Blood* **66**, 706-709

Lazarides, E. (1987) From genes to structural morphogenesis: the genesis and epigenesis of a red blood cell. *Cell* **51**, 345-356.

LeComte M. C., Feo C., Gautero H., Bournier O., Galand C., Garbarz M., Boivin P., Dhermy D. (1990a) Severe recessive poikilocytic anaemia with a new spectrin α chain variant. *Bri. J. Haematol.* **74**, 497-507.

LeComte, M.C., Gautero, H., Garbarz, M., Boivin, P., and Dhermy, D. (1990b) Abnormal tryptic peptide from the spectrin α- chain resulting from α or β-chain mutations: two genetically distinct forms of the Sp α$^{I/74}$ variant. *Brit. J. Haematol.* **76**, 406-413.

Lehnert, M.E. and Lodish, H.F. (1988) Unequal synthesis and differential degradation of α and β spectrin during murine erythroid differentiation. *J. Cell. Biol.* **107**, 413-426.

Leto, T.L. and Marchesi, V.T. (1984) A structural model of human erythrocyte protein 4.1. *J. Biol. Chem.* **259**, 4603-4608.

Li, X. And Bennett, V. (1996) Identification of the spectrin subunit and domains required for formation of spectrin/adducin/actin complexes. *J. Biol. Chem.* **271**, 15695-15702.

Litman, D., Hsu, C. J. and Marchesi, V. T. (1980) Evidence that spectrin binds to macromolecular complexes on the inner surface of the red cell membrane. *J. Cell Sci.* **42**, 1-22.

Liu, S-C, Zhai, S., Palek, J., Golan, D.E., Amato, D., Hassan, K., Babona D., Coetzer, T., Jarolim, P. et al (1990) Molecular defect of the band 3 protein in Souteast Asian ovalocytosis. *N Engl J Med* **323**, 1530-1538.

Liu, S-C., and Palek, J. (1984) Hemoglobin enhances the self-association of spectrin heterodimers in human erythrocytes. *J. Biol. Chem.* **259**, 11556-11562

Liu, S.-C. and Palek, J. (1980) Spectrin tetramer-dimer equilibrium and the stability of erythrocyte membrane skeletons, *Nature* **285**, 586-588.

Liu, S.-C., Derick, L.H. and Palek, J. (1987) Visualisation of the hexagonal lattice in the erythrocyte membrane skeleton. *J. Cell. Biol.* **104**, 527-536.

Liu, S.-C., Windisch, C.P., Kim, S. and Palek, J. (1984) Oligomeric states of spectrin in normal erythrocyte membranes: biochemical and electron microscopic studies. *Cell* **37**, 587-594.

Lodish, H., Baltimore, D., Berk, A., Zipursky, S.L., Matsudaira, P. and Darnell, J: chap22, Microfilaments: Cell mobility and control of cell shape, in Molecular cell biology 3rd edition,: W.H. Freeman and Company, New York, 1995, p991-1050.

Lu, P.W., Soong, C.-J. and Tao, M. (1985) Phosphorylation of ankyrin decreases its affinity for spectrin tetramer. *J. Biol. Chem.* **260**, 14958-14964.

Luna, E.J., Kidd, G.H. and Branton, D. (1979) Identification by peptide analysis of the spectrin-binding protein in human erythrocytes. *J. Biol. Chem.* **254**, 2526-2532.

Lux, S.E. (1979) Spectrin-actin membrane skeleton of normal and abnormal red blood cells. *Semin. Hematol.* **16**, 21-51.

Lux, S.E., John, K.M. and Bennett, V. (1990) Analysis of cDNA for human erythrocyte ankyrin indicates a repeat structure with homology to tissue-differentiation and cell-cycle control proteins. *Nature* **344**, 36-43.

Marchesi, V.T. and Steers, E.Jr. (1968) Selective solubilisation of a protein component of the red cell membrane. *Science*, **159**, 203-204.

Menhart, N., Mitchell, T., Lusitani, D., Topouzian, N. and Fung, L.W. (1996) Peptides with more than one 106-amino acid sequence motif are needed to mimic the structural stability of spectrin. *J. Biol. Chem.* **271**, 30410-30416.

Mische, S. M., Mooseker, M. S. and Morrow, J. S. (1987) Erythrocyte adducin: A calmodulin-regulated actin-bundling protein that stimulates spectrin-actin binding. *J. Cell. Biol.* **105**, 2837-2845.

Mische, S.M. and Morrow, J.S. (1989) Sp trin phosphorylation regualtes spectrin subunit interactions. *J. Cell. Biol.* **107**, 469a (abstr).

Morris, M. and Ralston, G.B. (1989) A thermodynamic model for the self-association of human spectrin. *Biochemistry* **28**, 8561-8567.

Morrow, J. S. and Marchesi, V. T. (1981) Self-assembly of Spectrin Oligomers *In vitro*: A Basis for a Dynamic Skeleton. *J. Cell. Biol.* **88**, 463-468.

Morrow, J. S., Speicher, D. W., Knowles, W. J., Hsu, J. C. and Marchesi, V. T. (1980) Identification of functional domains of human erythrocyte spectrin. *Proc. Natl. Acad. Sci. USA* 77, 6592-6596.

Mueller, T.J., Jackson, C.W., Dockter M.E. and Morrison, M. (1987) Membrane skeletal alterations during *in vivo* mouse red cell aging. Increase in the band 4.1a:4.1b ratio. *J. Clin. Invest* 79, 492-499.

Nehls., V., Drenckhahn, D., Joshi, R., Bennett, V. (1991) Adducin in erythrocyte precursor cells of rats and humans. *Blood* **78**, 1692-1696.

O'Farrell, P.H. (1975) High Resolution Two-Dimensional Electrophoresis of Proteins. *J. Biol. Chem.* **250**, 4007-4021.

Ohanian, V., Wolfe, L.C., John, K.M., Pinder, J.C, Lux, S.E. and Gratzer, W.B. (1984) Analysis of the ternary interaction of the red cell membrane skeletal proteins spectrin, actin, and 4.1. *Biochemistry* **23**, 4416-4420.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., Sekiya, T. (1989) Rapid and sensitive detection of polymorphisms of human DNA by gel electrophoresis as single-stranded conformational polymorphisms. *Proc. Natl. Acad. Sci. USA,* **86**, 2766-2770.

Padgett, R. A., Grabowski, P. J., Konarska M. M., Seiler, S. and Sharp, P.A. (1986) Splicing of messenger RNA precursors. *Annu. Rev. Biochem.* **55**, 1119-1150.

Palek, J. (1986) Hereditary elliptocytosis, spherocytosis and related disorders: Consequences of a deficiency or a mutation of membrane skeletal proteins. *Blood Reviews* **1**, 147-168.

Palek, J. (1995) The red cell membrane. Williams Hematology, 5th edition, chapter 37, pp406-417. Edited by Beutler, E., Litchman, M.A., Coller, B.S. And Kipps, T.J., McGraw-Hill, New York.

Palek, J. and Jarolim, P. (1995) Hereditary spherocytosis, elliptocytosis, and related disorders. Williams Hematology, 5th edition, chapter 52, pp 536-556. Edited by Beutler, E., Litchman, M.A., Coller, B.S. And Kipps, T. J., McGraw-Hill, New York.

Parry, D.A., Dion, T.W. and Cohen, C. (1992) Analysis of the three-α-helix motif in the spectrin superfamily. *Biophys. J.* **61**, 858-867.

Pascual, J., Pfuhl, M., Rivas, G., Pastore, A. And Saraste, M. (1996) The spectrin repeat folds into a three-helix bundle in solution. *FEBS Lett* **383**, 201-207.

Pascual, J., Pfuhl, M., Walther, D., Saraste, M. and Nilges, M. (1997) Solution structure of the spectrin repeat: a left handed antiparallel triple-helical coiled-coil. *J Mol Biol* **273**, 740-751.

Peters, L.L. and Lux, S.E. (1993) Ankyrins: structure and function in normal cells and hereditary spherocytosis. *Semin. Hematol.* **30**, 85-118.

Peters, L.L., Shivdasani, R.,A., Liu, S.-C., Hanspal, M., John, K.M., Gonzalez, J.M., Brugnara, C., Gwynn, B., Mohandas, N., Alper, S.L., Orkin, S.H. and Lux, S.E. (1996) Anion exchanger 1 (band 3) is required to prevent erythrocyte membrane loss but not to form the membrane skeleton. *Cell* **86**, 917-927.

Pinder, J.C. and Gratzer, W. B. (1983) Structural dynamic states of actin in the erythrocyte. *J Cell Biol* **96**, 768-775.

Pinder, J.C., Ungewickell, E., and Bray, D. and Gratzer (1978) The spectrin actin complex and erythrocyte shape. *J. Supramol. Struct.* **8**, 439-445.

Prchal, J.T., Norley, B.J. and Yoon, S.H. (1987) Isolation and characterisation of cDNA clones for human erythrocyte β spectrin. *Proc. Natl. Acad. Sci. USA* **84**, 7468-7472.

Prchal, J.T., Yoon, S.H., Norley, B.J., Coetzer, T.L. and Palek, J. (1986) Isolation of β spectrin clones from a human reticulocyte cDNA library: β spectrin is on chromosome **14**. *Blood* 68 supl 1, 39a.

Ralston, G.B. (1978) Physical-chemical studies of spectrin. *J Supramol. Struct.* **8**, 361-370.

Ren, R., Mayer, B.J., Cicchetti, P. and Baltimore, D. (1993) Identification of a ten-amino acid proline-rich SH3 domain binding site. *Science* **259**, 1157-1161.

Ruff, P., Speicher, D.W. and Husain-Chisti, A. (1991) Molecular identification of a major palmitoylated erythrocyte membrane protein containing the src homology 3 motif. *Proc. Natl. Acad. Sci. USA* **88**, 6596-6599.

Sahr, K.E., Coetzer, T.L., Moy, L.S., Derick, L.H., Chisti, A.H., Jarolim, P., Lorenzo, F., Miraglia del Guidice, E., Iolascon, A., Gallenello, R., Cao, A., Delaunay, J., Liu, S-C and Palek, J. (1993) Spectrin Cagliari: an ala→gly substitution in helix 1 of the β spectrin repeat 17 that severely disrupts the structure and self-association of the spectrin heterodimer. *J. Biol. Chem.* **268**, 22656-22662.

Sahr, K.E., Laurila, P., Kotula, L., Scarpa, A.L., Coupal, E., Leto, T.L., Linnenbach, A.J., Winkelmann, J.C., Speicher, D.W., Marchesi, V.T., Curtis, P.J. and Forget, B.G. (1990) The complete cDNA and polypeptide sequences of human erythroid α-spectrin. *J. Biol. Chem.* **265**, 4434-4443.

Sheetz, M. P. (1979) Integral membrane protein interaction with triton cytoskeletons of erythrocytes. *Biochem. Biophys. Acta* **557**, 122-134.

Shen, B.W., Josephs, R. and Steck, T.L. (1986) Ultrastructure of the intact skeleton of the human erythrocyte membrane. *J. Cell. Biol.* **102**, 997-1006.

Shotton, D.M., Burke, B. and Branton, D. (1979) The molecular structure of human erythrocyte spectrin. Biophysical and electron microscopic studies. *J. Mol. Biol.* **131**, 303-329.

Siegel, D. L. and Branton, D. (1985) Partial purification and characterization of an actin-bundling protein, band 4.9, from human erythrocytes. *J. Cell. Biol.* 100, 775-785.

Speicher, D. W, DeSilva, T. M., Speicher, K. D., Ursitti, J. A., Hembach P. and Weglarz, L. (1993) Location of the Human Red Cell Spectrin Tetramer Binding Site and Detection of a Related "Closed" Hairpin Loop Dimer Using Proteolytic Footprinting. *J. Biol. Chem.* **268**, 4227-4235.

Speicher, D. W. and Marchesi, V. T. (1982) Spectrin domains: Proteolytic susceptibility as a probe of protein structure. *J. Cellular Biochemistry* **18**, 479-492.

Speicher, D. W., and Marchesi, V. T. (1984) Erythrocyte spectrin is comprised of many homologous tripical helical segments. *Nature* **311**, 177-180.

Speicher, D. W., Morrow, J.S., Knowles, W.J. and Marchesi, V.T. (1980) Identification of proteolytically resistant domains of human erythrocyte spectrin. *Proc. Natl. Acad. Sci. USA* 77, 5673-5677.

Speicher, D. W., Morrow, J.S., Knowles, W.J. and Marchesi, V.T. (1982) A structural model of human erythrocyte spectrin *J. Biol. Chem.* **257**, 9093-9101.

Speicher, D. W., Weglarz, L. and DeSilva, T. M. (1992) Properties of Human Red Cell Spectrin Heterodimer (Side-to-Side) Assembly and Identification of an Essential Nucleation Site. *J. Biol. Chem.* **267**, 14775-14782.

Speicher, D.W. and Ursitti, J.A. (1994) Conformation of a mammoth protein. *Current Biology* **4**, 154-157.

Sykes, B.C. (1983) DNA in Inheritable diseases. *Lancet.* **II**, 787-788.

Tanner, M.J.A., Bruce, L., Martin, P.G. (1991) Melanesian hereditary ovalocytes have a deletion in red cell band 3. *Blood* **78**, 2785-2786.

Till, J.E. and McCulloch, (1980) Hemopoietic stem cell differentiation. *Biochem. Biophys. Acta* **605**, 431-459.

Tse, W. T., LeComte, M-C., Costa, F.F., Garbarz, M., Feo, C., Boivin, P., Dhermy, D. and Forget, B. (1990) Point Mutation in the β-spectrin Gene Associated with αI/74 Hereditary Elliptocytosis. *J. Clin. Invest* **86**, 909-916.

Ungewickell, E. and Gratzer, W. (1978) Self-Association of Human Spectrin. *Eur. J. Biochem.* **88**, 379-385.

Ursitti, J.A., Kotula, L., DeSilva, T.M., Curtis, P.J. and Speicher, D.W. (1996) Mapping the human erythrocyte beta-spectrin dimer initiation site using recombinant

peptides and correlation of its phasing with the alpha-actinin dimer site. *J. Biol. Chem.* **271**, 6636-6644.

Wasenius, V.M, Saraste, M., Salven, P., Eramaa, M., Holm, M., Lehto, V.P. (1989) Primary structure of the brain alpha-apectrin. *J. Cell. Biol.* **108**, 79-93.

Weaver, D.C., Pasternack, G.R. and Marchesi, V.T. (1984) The structural basis of ankyrin function. *J. Biol. Chem.* **259**, 6170-6175.

Weiss, L. (1983) The red pulp of the spleen: Structural basis of blood flow. *Clin. Haematol.* **12**, 375-393.

Williamson, M.P. (1994) The structure and function of proline-rich regions in proteins. *Biochem. J.* **297**, 249-260.

Winkelmann, J.C. and Forget, B.G.(1993) Erythroid and non-erythroid spectrins. *Blood* **81**, 3173-3185.

Winkelmann, J.C., Chang, J-C., Tse, W.T., Scarpa, A.L., Marchesi, V.T. and Forget, B.G. (1990) Full-length Sequence of the cDNA for Human Erythroid β-Spectrin *J. Biol. Chem.* **265**, 11827-11832.

Winkelmann, J.C., Chang, J-C., Tse, W.T., Scarpa, A.L., Marchesi, V.T. and Forget, B.G. (1990) Full-length sequence of the cdna for human erythroid β-spectrin. *J. Biol. Chem.* **265**, 11827-11832.

Winograd, E., Hume, D. and Branton, D. (1991) Phasing the conformational unit of spectrin. *Proc. Natl. Acad. Sci. USA* **88**, 10788-10791.

Woods, C.M., Boyer, B., Vogt, P.K. (1986) Control of erythroid differentiation. Asynchronous expression of the anion transporter and the peripheral components of the membrane skeleton in AEV- and S13 transformed cells. *J. Cell. Biol.* **103**, 1789-1798.

Yan, Y., Winograd, E., Viel, A., Cronin, T., Harrison, S. C. and Branton, D. (1993) Crystal Structure of the Repetitive Segments of Spectrin. *Nature* **262**, 2027-2029.

Yoshino, H., and Marchesi, V.T. (1984) Isolation of Spectrin Subunits and Reassociation *in vitro*. *J. Biol. Chem.* **259**, 4496-4500.

Yoshino, H., and Marchesi, V.T. (1985) Interaction between the subunits of human erythrocyte spectrin using a fluorescence probe. *Biochem. Biophys. Acta* **812**, 786-792.

Yu, J. And Goodman, S.R. (1979) Syndeins: The spectrin-binding protein(s) of the human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA* **76**, 2340-2344.

Yu, J., Fischman, D.A. and Steck, T.L. (1973) Selective solubilization of proteins and phospholipids from red blood cell membranes by non-ionic detergent. *J. Supramol. Struct.* **1**, 233-248.

Zail, S. S. and Coetzer, T. L. (1984) Defective Binding of Spectrin to Ankyrin in a Kindred with Recessively Inherited Hereditary Elliptocytosis *J. Clin. Invest* **74**, 753-762.

Author Burke J P W G
Name of thesis Spectrin Johannesburg: A And B Spectrin Defects In A South African Kindred With Hereditary Elliptocytosis Burke J P W G 1998

PUBLISHER:
University of the Witwatersrand, Johannesburg
©2013

LEGAL NOTICES:

Copyright Notice: All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed, or otherwise published in any format, without the prior written permission of the copyright owner.

Disclaimer and Terms of Use: Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page) for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.