Mapping of a spectrin-binding domain of human erythrocyte membrane protein 4.2

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Protein 4.2 is a major component of the red blood cell membrane skeleton. Deficiency of protein 4.2 is linked with a variety of hereditary haemolytic anaemias. However, the interactions of protein 4.2 with other proteins of the erythrocyte membrane remain poorly understood. The major membrane-binding site for protein 4.2 resides on the cytoplasmic domain of band 3. Protein 4.2 interacts directly with spectrin in solution, suggesting that it stabilizes interactions between the membrane skeleton and the erythrocyte membrane. A 30 kDa polypeptide, with its N-terminus corresponding to amino acid residue 269, derived by partial proteolysis of protein 4.2, was found to interact with biotinylated spectrin in gel renaturation assays. A series of overlapping glutathione S-transferase fusion peptides were constructed, and an α-helical domain encompassing residues 470–492 was found to be instrumental in mediating protein 4.2–spectrin interactions. Direct binding of a synthetic peptide, with the sequence corresponding to residues 470–492, to spectrin and the ability of the peptide to inhibit spectrin binding of protein 4.2 confirmed that these residues are crucial in mediating protein 4.2–spectrin interactions.

Key words: cytoskeleton, protein interaction, red blood cell.

INTRODUCTION

In the red blood cell, the flexible and elastic meshwork of proteins underlying the lipid bilayer comprises the cytoskeleton and is responsible for maintaining the biconcave shape of the erythrocyte, for its reversible deformability and for membrane structural integrity [1–3]. Hereditary haemolytic anaemias represent conditions where either the vertical protein interactions, which ensure the anchoring of the skeleton to the lipid bilayer, or the horizontal protein interactions, which account for the lateral deformability and extensibility of the skeleton, are compromised, due to defects and deficiencies in one or more of the membrane proteins that participate in this complex network of interactions. Protein 4.2 is a 72 kDa peripheral membrane protein that comprises approx. 5 % of the total membrane protein [4–7]. It is present in $\approx 2 \times 10^5$ copies/red cell [8]. Protein 4.2 deficiency results in overt haemolysis, anaemia and a borderline variety of hereditary spherocytosis [9–13], which may be due to either a primary defect in protein 4.2 or defects in some other proteins, primarily band 3 with which it associates. Protein 4.2-null mice show mild spherocytosis [14]. Band 3–protein 4.2–ankyrin–spectrin interactions probably stablilize linkages between the cytoskeleton and the overlying membrane. Protein 4.2 associates *in vitro* with band 3 [15,16], ankyrin [16] and spectrin [17]. Human erythrocyte protein 4.2 is N-terminally myristoylated [18] and palmitoylated [19], and its interaction with band 3 is modulated by palmitoylation [15]. It can be phosphorylated by a red-cell-membrane kinase that partially co-purifies with it, and has properties similar to the catalytic subunit of cAMP-dependent kinase [20]. In addition, protein 4.2 shares a conserved 11-amino acid motif with dematin, which contains an ATP-binding P-loop [21]. Since band 3–protein 4.2–spectrin interactions play a role in stabilizing membrane–skeleton interactions, we have mapped the spectrin-binding site on protein 4.2.

EXPERIMENTAL

Materials

Staphylococcal V8 protease, Iodobeads iodination reagent and *N*-hydroxysuccinimidoiminobiotin hydrobromide (NHS-iminobiotin) were from Pierce Chemical Co., Rockford, IL, U.S.A. Synthetic peptide J3, GSLQKEVLERVEKEKMEREKDN (where single-letter amino-acid notation has been used), corresponding to residues 470–492 of protein 4.2, was purchased from Mimotopes Pty Ltd, Victoria, Australia, and streptavidin–agarose was from Invitrogen, Carlsbad, CA, U.S.A. All other reagents were of analytical grade.

SDS/PAGE

Protein samples were separated on the basis of their molecular mass by SDS/PAGE as described by Laemmli [22].

Electroblotting on to PVDF and N-terminal sequencing

Electroblotting on to PVDF membranes was carried out as described by Matsudaira [23]. N-terminal sequencing was performed on an Applied Biosystems model 477A Pulsed Liquid Sequenator with on-line analysis of the phenylthiohydantoin amino acids by HPLC at the protein sequencing facility of Eurosequence, Gröningen, The Netherlands.

Radio-iodination of protein 4.2

Protein 4.2 was purified from human erythrocytes as described previously [19]. Purified protein 4.2 (1 mg) was iodinated using the Iodobeads iodination reagent and $Na^{125}I$ (1 mCi; Amersham Biosciences, Little Chalfont, Bucks., U.K.), as recommended by

Abbreviations used: GST, glutathione S-transferase; PBS-T, PBS containing 0.5 % Tween; streptavidin-POD, streptavidin-linked H_2O_2 oxidoreductase.

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Table 1 Design of recombinant protein 4.2-derived peptides

Amino acid numbering has been performed according to the numbering of the codons specified by Sung et al. [27]

Peptide	Amino acids	5′ Primer	3′ Primer
E1	269–325	5′-CCGGATCCATGGGGCCTTGCTGAACAA-3′	5′-AAAGAATTCTGAGGCAAACGTGGTCAC-3′
E2	269–421	5′-CCGGATCCATGGGGCCTTGCTGAACAA-3′	5′-AAAGAATTCGGCATTTATGGCAGCAA-3′
E3	269–522	5′-CCGGATCCATGGGGCCTTGCTGAACAA-3′	5′-AAAGAATTCCTGGGCATCCCCTCTCAG-3′
H1	370–450	5′-CCGGATCCATTTGCCCCAGGGTTATGAT-3′	5′-AAAGAATTCGGTGCTGATGTTGTTGCC-3′
H2	370–470	5′-CCGGATCCATTTGCCCCAGGGTTATGAT-3′	5′-AAAGAATTCCCTTCAGGATACTTGTA-3′
H3	370–492	5′-CCGGATCCATTTGCCCCAGGGTTATGAT-3′	5′-AAAGAATTCGTTGTCTTTCTCACGTTC-3′
H4	370–522	5′-CCGGATCCATTTGCCCCAGGGTTATGAT-3′	5′-AAAGAATTCCTGGGCATCCCCTCTCAG-3′
G1	410–450	5′-CCGGATCCATACCCCAGCAGTGTCAGAC-3′	5′-AAAGAATTCGGTGCTGATGTTGTTGCC-3′
G2	410–470	5′-CCGGATCCATACCCCAGCAGTGTCAGAC-3′	5′-AAAGAATTCCCTTCAGGATACTTGTA-3′
G3	410–492	5′-CCGGATCCATACCCCAGCAGTGTCAGAC-3′	5′-AAAGAATTCGTTGTCTTTCTCACGTTC-3′
G4	410–522	5′-CCGGATCCATACCCCAGCAGTGTCAGAC-3′	5′-AAAGAATTCCTGGGCATCCCCTCTCAG-3′

the manufacturer, and dialysed overnight against 5 mM sodium phosphate buffer/0.5 mM EGTA/0.5 mM dithiothreitol/120 mM KCl (pH 8).

Biotinylation of peptide J3 and spectrin

Spectrin was purified as described by Bennett [24]. Purified spectrin or peptide J3 (5 mg/ml) was taken in 500 μl of 0.1 M Hepes (pH 8.0) and treated with 50–100-fold molar excess of NHS-iminobiotin dissolved to 1 mg/ml in DMSO. After incubation on ice for 2 h, biotinylated spectrin or peptide was dialysed against the appropriate buffer.

Blot-overlay assay

This assay was performed as described by Platt et al. [25]. Protein 4.2 or recombinant protein 4.2-derived glutathione S-transferase (GST) fusion proteins were electrophoresed on SDS/polyacrylamide gels. The proteins were electrophoretically transferred on to nitrocellulose paper. The blot was then blocked with blocking buffer [150 mM NaCl/10 mM sodium phosphate buffer (pH 7.5)/0.05 % (v/v) Tween 20 containing 3 % (w/v) gelatin] for 30 min at 25 °C. The blotted proteins were allowed to renature in renaturing buffer [150 mM NaCl/10 mM sodium phosphate buffer (pH 7.5)/0.05 % (v/v) Tween 20/40 mg/ml BSA] for 1 h at 25 °C. After thorough washing with ligand-blot buffer [150 mM NaCl/10 mM sodium phosphate buffer (pH 7.5)/0.05 % (v/v) Tween 20], the blot was incubated with 1 μM biotinylated spectrin for 1 h at 25 °C. As controls, similar blots were incubated in buffer alone without any spectrin for 1 h. After washing the blots with ligand-blot buffer, membranes were incubated with streptavidin-linked H_2O_2 oxidoreductase (streptavidin-POD), and detection was carried out using the BM Chemiluminescence Blotting Kit (Biotin/Streptavidin) from Roche Molecular Biochemicals Mannheim, Germany.

Construction of recombinant proteins

To produce recombinant proteins, defined domains of protein 4.2 were amplified from protein 4.2 cDNA in the vector pGEM3z by PCR. The primers for the constructs are given in Table 1. The PCR-amplified products were purified, cloned in the vector pUC19, sequenced using the Thermosequenase cycle sequencing kit from Amersham Biosciences, and finally transferred to the vector pGEX-3X using asymmetric *Bam*HI and *Eco*RI sites present in the PCR products.

Expression and purification of fusion proteins

Recombinant fusion proteins were expressed in *Escherichia coli* BL21 or *E. coli* DH5α. Expression of protein was carried out in the presence of 0.1 mM isopropyl β-D-thiogalactoside at 30 °C. After harvesting the cells, the cell pellet was suspended in 50 mM Tris/HCl (pH 8)/5 mM EDTA/50 mM NaCl containing 0.15 mM PMSF, 1 mM pefabloc, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 mM 2-mercaptoethanol and 1 % (v/v) Triton X-100, and disrupted by sonication on a Labsonic 2000 sonicator (B. Braun, Melsungen, Germany) for a maximum of 10 s, avoiding frothing. Recombinant proteins E1, E2 and E3 were present in the post-sonicate pellet. The post-sonicate pellet was extracted with 50 mM Tris/HCl (pH 8) containing 5 M urea, 0.15 mM PMSF, 1 mM pefabloc, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 5 mM 2-mercaptoethanol using a Dounce homogenizer and incubated for 2 h on ice. The mixture was centrifuged at 100000 ***g*** for 1 h and the supernatant was dialysed for 3 h at 4 °C against renaturation buffer [126.6 mM NaCl/10 mM sodium phosphate buffer (pH 7.3) containing 5 mM 2-mercaptoethanol, 5 mM EDTA, 20 % (v/v) glycerol, 1 % (v/v) Triton X-100, 0.15 mM PMSF, 1 mM pefabloc, 0.1 μg/ml leupeptin and 0.1 μg/ml pepstatin]. This was followed by dialysis overnight against 126.6 mM NaCl/10 mM sodium phosphate buffer/5 mM EDTA/0.15 mM PMSF/1 % Triton X-100 (pH 7.3). Renatured fusion proteins were purified from the urea extract by affinity chromatography on glutathione–Sepharose, as described by the manufacturers. The recombinant proteins G1, G2, G3, G4, H1, H2, H3 and H4 were present in the post-sonicate supernatant and were purified by loading the supernatant on glutathione–Sepharose.

Binding assay for studying the interaction between protein 4.2-derived recombinant polypeptides and spectrin

Proteins were adsorbed to the wells of a microtitre ELISA plate. Adsorption was allowed to proceed for 12 h at 4 °C. The wells were washed with PBS containing 0.5 % Tween (PBS-T). Non-specific binding sites were blocked with ELISA blocking buffer [1 % (w/v) BSA in PBS-T] for 1 h at 37 °C. The wells were washed extensively with PBS-T. Biotinylated spectrin [100 μl; 0.2–10 μM] was added to each well and the binding was allowed to proceed for 6 h at 25 °C. The wells were then washed extensively with PBS-T. Binding of biotinylated spectrin was measured by adding 0.1 μg/ml (in 100 μl) streptavidin–alkaline phosphatase to each well and incubating for 60 min at 25 °C. This was followed by colour development by the addition of 1 mg/ml *p*-nitrophenyl phosphate prepared in 0.1 M glycine

containing 0.01 M $MgCl_2$ (pH 10.4). Absorbance was measured at 405 nm. In each case, control experiments were performed in which equivalent amounts of BSA were first adsorbed to the wells. Controls were also run using recombinant GST alone.

Analysis of the interaction between protein 4.2-derived recombinant polypeptides with spectrin in solution

Spectrin was mixed with various GST fusion peptides in a 50 μl reaction volume in buffer A [120 mM KCl/5 mM sodium phosphate buffer (pH 8), /0.1 mM EGTA/20 μg/ml PMSF/0.5 mM dithithreitol/1 mg/ml gelatin], and incubated for 6 h at 25 °C. The spectrin bound to the fusion peptide was precipitated by the addition of 10 μl of washed glutathione–Sepharose beads to the reaction mixture. The pellet was washed, boiled for 5 min in SDS/PAGE-denaturing buffer and subjected to SDS/PAGE. This was followed by electrophoretic transfer of the separated proteins on to nitrocellulose membranes, blocking with 5% (w/v) non-fat dried milk and incubation of the membranes with an anti-spectrin antibody [26] for 1 h at 25 °C. Membranes were then incubated with secondary antibody [goat anti-(rabbit IgG) conjugated to horseradish peroxidase] for 1 h at 25 °C and spectrin was detected by using the BM Chemiluminescence Blotting Kit (Roche Molecular Biochemicals).

Binding of spectrin to peptide J3

Biotinylated peptide was incubated with purified spectrin in buffer A for 6 h at 25 °C in a volume of 100 μl. A control reaction was set up without any peptide. After incubation, 10 μl (50% slurry) of streptavidin–agarose was added to each tube, followed by incubation for 30 min at 25 °C. The suspension was centrifuged, the pellet was washed twice with buffer A, boiled with SDS/PAGE-denaturing buffer and separated by SDS/PAGE. Proteins were then transferred electrophoretically on to nitrocellulose, followed by detection of peptide-associated spectrin as described above. Peptide-binding assays were also carried out on streptavidin-coated microtitre plates. Plates were incubated overnight at 4 °C with biotinylated peptide J3. Non-specific binding sites were blocked with ELISA blocking buffer as described above, followed by incubation with spectrin (0.2–10 μM) at various concentrations for 6 h at 25 °C. Wells were washed extensively. Bound spectrin was quantified by successive incubations with an anti-spectrin antibody, an alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibody and colour development with *p*-nitrophenyl phosphate. In each case controls were run in wells where no peptide had been added.

Competitive inhibition of protein 4.2–spectrin association by peptide J3

Competitive inhibition by peptide J3 of the association of protein 4.2 with biotinylated spectrin was studied by allowing ^{125}I-protein 4.2 to interact with biotinylated spectrin (20 μg) in a volume of 100 μl of buffer A for 6 h at 25 °C in the absence or presence of different concentrations of the peptide. Streptavidin–agarose (10 μl of 50% slurry) was added to each tube, followed by incubation for 30 min at 25 °C. The suspension was centrifuged at 2000 ***g***, the pellet was washed twice with 100 μl of buffer A and the radioactivity was counted on a γ-counter. Control experiments, lacking biotinylated spectrin, were run in parallel to account for non-specific binding.

RESULTS

Partial proteolysis of labelled protein 4.2 and blot-overlay assay

Purified and electroeluted protein 4.2 was partially proteolysed with staphylococcal V8 protease and loaded in separate lanes of a denaturing SDS/polyacrylamide gel. After running the gel, one lane was transferred on to a nitrocellulose membrane (for blot-overlay assay) and the proteins of a second lane were transferred on to a PVDF membrane (for N-terminal sequencing).

The proteins transferred on to nitrocellulose were renatured as described previously [25]. After incubation with blocking buffer to block non-specific binding, the renatured proteins were incubated with biotinylated spectrin followed by development using chemiluminescence. Protein 4.2 (Figure 1, lane B), and a 30 kDa band derived from it following proteolysis with the V8 protease (Figure 1, lane C), gave positive bands. This 30 kDa polypeptide was therefore likely to represent a spectrin-interacting domain. No positive band was detected in control blots, which were incubated in buffer without any spectrin (Figure 1, lanes D and E). The 30 kDa polypeptide blotted on to a PVDF membrane was subjected to N-terminal sequencing, and the sequence obtained was ^{269}Gly-Ala-Leu-Leu-Asn-Lys-Arg-Arg-Gly-Ser.

Expression of recombinant GST fusion proteins

Expression of fusion proteins in *E. coli* was chosen as a strategy for studying the binding of a series of protein 4.2-derived polypeptides, commencing from position 269, with spectrin. Table 1 lists the different domains expressed as GST fusion proteins. The amino acid spans of the different recombinants are depicted diagrammatically in Figure 2. Recombinant proteins spanning residues 269–522 were purified by affinity chromatography (Figure 3). Recombinant proteins spanning regions further towards the C-terminus could not be purified successfully.

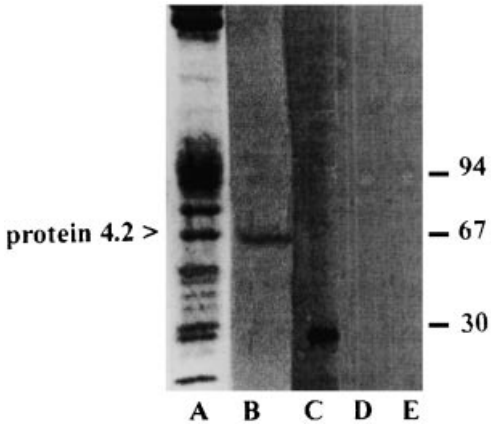

Figure 1 Analysis of the spectrin-interacting domain of protein 4.2

Erythrocyte ghosts (lanes A and B) or peptides of protein 4.2 derived by partial proteolysis with V8 protease (lanes B–E) were run on SDS/polyacrylamide gels and either stained with Coomassie Blue (lane A) or transferred on to nitrocellulose, renatured, incubated in the absence (lanes D and E; controls for samples in lanes B and C respectively) or presence (lanes B and C) of biotinylated spectrin. Interaction of protein 4.2 (lane B) or the peptides derived from protein 4.2 following V8 protease digestion (lane C) with biotinylated spectrin was detected by chemiluminsecence. Molecular-mass markers (in kDa) are indicated on the right.

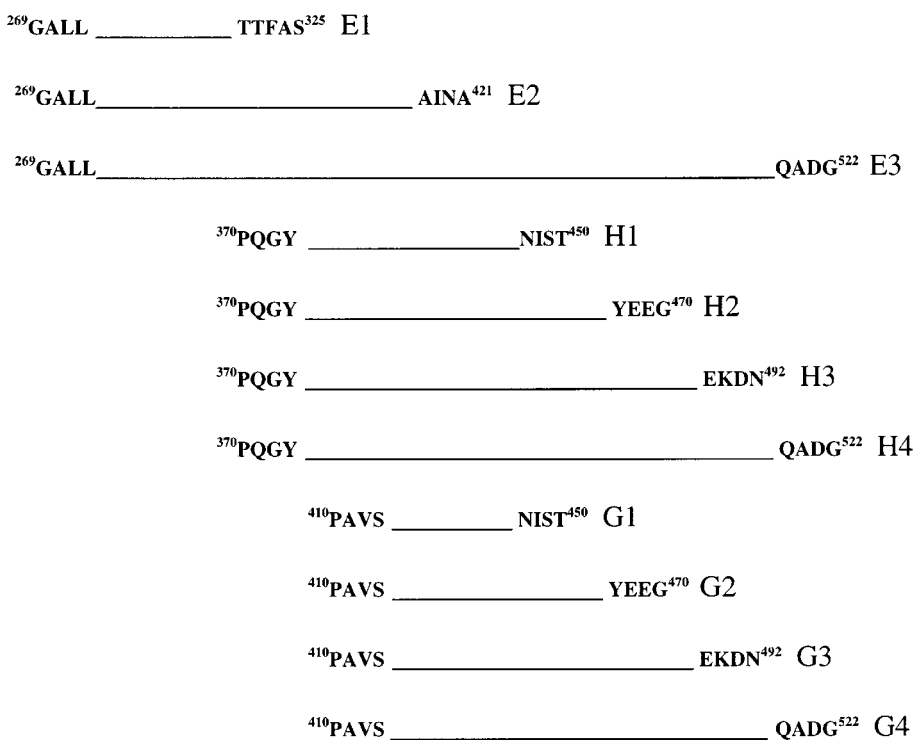

Figure 2 Diagrammatic representation of the recombinant protein 4.2-derived peptides

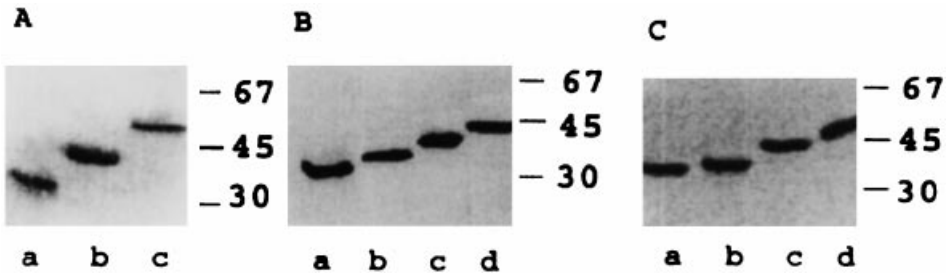

Figure 3 Purification of the recombinant fusion proteins

Coomassie Brilliant Blue-stained SDS/polyacrylamide gels of the fusion proteins purified on glutathione–Sepharose 4B. (**A**) Fusion proteins E1 (lane a), E2 (lane b) and E3 (lane c) (**B**) Fusion proteins G1 (lane a), G2 (lane b), G3 (lane c) and G4 (lane d). (**C**) Fusion proteins H1 (lane a), H2 (lane b), H3 (lane c) and H4 (lane d). Molecular-mass markers (in kDa) are indicated on the right of each panel.

Binding of recombinant fusion proteins with biotinylated spectrin

Recombinant proteins E1, E2 and E3 were first analysed with respect to their spectrin-binding ability. E3 bound to spectrin, whereas binding was too low to be quantified in the case of E2 and E1. The region between residues 269–522 appeared to play an important role in spectrin binding. Scatchard analysis of the binding of E3 with spectrin was performed. The Scatchard plot appeared linear, suggesting a single class of binding site (results not shown). The K_d value was determined to be 0.65 μM (Table 2). On the basis of this observation, further recombinant proteins were chosen for determination of their spectrin-binding abilities. It was observed that recombinant proteins H3, H4, G3 and G4 bound to spectrin with an affinity comparable with that of E3 (Table 2). On the other hand, recombinant proteins H1, H2, G1 and G2 did not possess measurable spectrin-binding ability. Non-specific binding (i.e. readings obtained for BSA or GST) was less than 10%. A representative Scatchard analysis of the binding of G3 to spectrin is shown in Figure 4.

The interaction of spectrin with recombinant fusion peptide was also shown in solution. After incubation of recombinant fusion proteins with purified spectrin, the complex was pulled-down with glutathione–Sepharose, followed by electrophoretic separation and detection as described above. As shown in Figure 5(A), G3 was able to bind biotinylated spectrin in solution, whereas G2 did not show any detectable binding. No colour development was observed in the control with recombinant GST alone, suggesting that non-specific interactions between GST and spectrin were not responsible for the colour development observed with the recombinant fusion protein G3.

Table 2 K_d values for binding of recombinant protein 4.2-derived peptides with spectrin

Results represent the means of five separate experiments. Figures in parantheses represent the range of values obtained. –, the binding was too low and reliable estimates of K_d were not possible.

Recombinant protein	K_d (μM)
E1	–
E2	–
E3	0.65 (0.55–0.77)
H1	–
H2	–
H3	0.60 (0.57–0.75)
H4	0.59 (0.50–0.65)
G1	–
G2	–
G3	0.70 (0.60–0.80)
G4	0.69 (0.60–0.80)

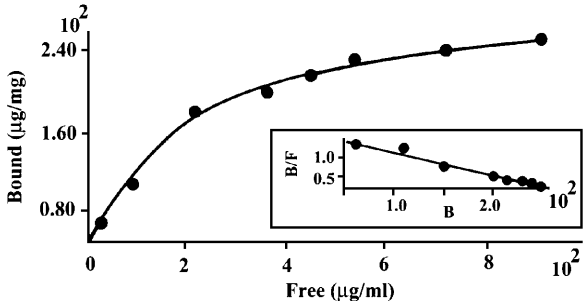

Figure 4 Scatchard analysis of the interaction of G3 with spectrin

The binding of G3 with biotinylated spectrin was assayed in microtitre plates. Results were analysed using the non-linear regression curve-fitting software ENZFITTER (Elsevier Biosoft, Cambridge, U.K.).

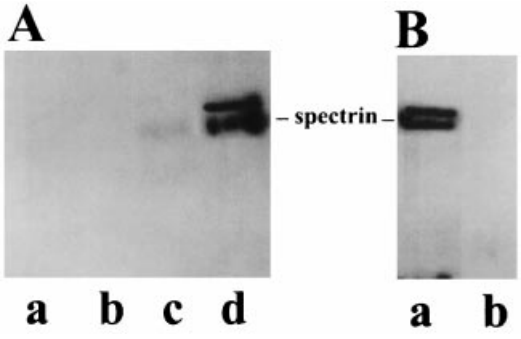

Figure 5 Interaction of spectrin with protein 4.2-derived fusion proteins/peptides in solution

(**A**) Spectrin was incubated in the absence (lane a) or presence of GST (lane b), G2 (lane c) or G3 (lane d), followed by precipitation of the complex with glutathione–Sepharose. Bound spectrin was detected using an anti-spectrin antibody and HRP-conjugated secondary antibody as described in the Materials and methods section. (**B**) Spectrin was incubated in the absence (lane a) or presence (lane b) of biotinylated peptide J3, the complex was precipitated using streptavidin–agarose. Bound spectrin was detected as described in the Experimental section.

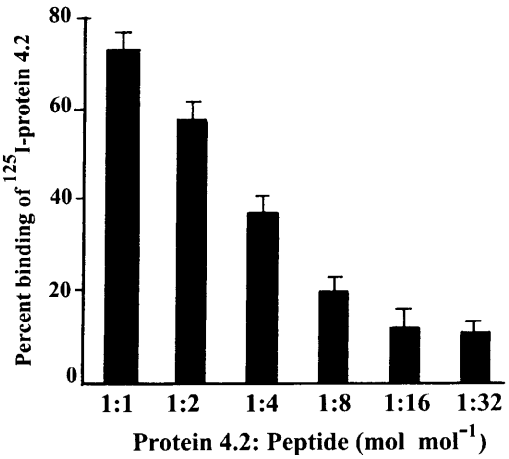

Figure 6 Inhibition of the interaction of ^{125}I-protein 4.2 with biotinylated spectrin by peptide J3

^{125}I-Protein 4.2 was allowed to react with biotinylated spectrin for 6 h at 25 °C in the absence or presence of various amounts of peptide J3, as described in the Experimental section. The binding of protein 4.2 was determined after precipitation of the protein 4.2–spectrin complex with streptavidin–agarose. The binding of protein 4.2 in the absence of peptide was taken to be 100% and the percentage of binding was calculated relative to this. Results are means ± S.D. of three separate determinations.

Spectrin binding to peptide J3

Peptide J3, encompassing residues 470–492 of protein 4.2, was biotinylated and incubated with spectrin for 6 h at 25 °C. Association of peptide J3 with spectrin was visualized by pulling-down the complex with streptavidin–agarose, followed by electrophoretic separation and detection as described above. Peptide J3 was found to interact with spectrin (Figure 5B). Binding of spectrin to immobilized biotinylated peptide J3 was also analysed. The binding occurred with a K_d value of 0.7 μM (mean of three determinations; values ranged from 0.66–0.74 μM), suggesting that the region encompassed by residues 470–492 of protein 4.2 was probably sufficient for the binding of protein 4.2 with spectrin.

Inhibition of the interaction of ^{125}I-protein 4.2 with biotinylated spectrin by peptide J3

The inability of G2 or H2 to bind spectrin indicated that residues 470–492 were crucial for binding. In conformity with this, it was found that the interaction of native protein 4.2 with spectrin was inhibited by peptide J3 (Figure 6). Inhibition (90%) was obtained at a peptide:protein 4.2 molar ratio of 1:16. Non-specific binding obtained in the control (without spectrin) was less than 10%.

DISCUSSION

Protein 4.2 binds to the cytoplasmic domain of band 3, which serves as a site for anchorage of several membrane skeletal proteins. This makes it likely that protein 4.2 plays a role in promoting membrane–skeleton linkages. Protein 4.2 has been shown to bind to spectrin in solution and to promote the binding

protein 4.2 (human erythrocyte)	478	E	K	E	K	M	E	R	E	K	D	487
porcine radixin	336	E	K	E	R	I	E	R	E	K	E	345
human radixin	336	E	K	E	R	I	E	R	E	K	E	345
murine ezrin	336	E	K	E	Q	M	L	R	E	K	E	345
bovine ezrin	336	E	K	E	Q	M	M	R	E	K	E	345
		*	*	*	·	·		*	*	*	·	

Figure 7 Multiple sequence alignment of domains of human erythrocyte protein 4.2, ezrin and radixin

Alignment of the amino acids was performed using the BLAST algorithm. An asterisk indicates an identical residue and a bold point a conservative substitution.

of spectrin to ankyrin-depleted inside-out vesicles, suggesting that protein 4.2 interacts directly with spectrin [17]. Considering that protein 4.2 is one of several proteins that stabilize linkages between the membrane skeleton and erythrocyte membrane, the spectrin-interacting domain of protein 4.2 has been mapped. Partial proteolysis of protein 4.2, followed by a blot-overlay assay, identified the N-terminal 30 kDa domain of protein 4.2 as the spectrin-binding domain. The region encompassing residues 269–522, expressed as a GST fusion protein, was found to interact with spectrin with moderate affinity. The interaction was saturable, and the Scatchard analysis showed the presence of a single class of binding site. This region includes: (1) the hydrophobic stretch between residues 293–325, which bears the highest sequence similarity to human placental factor XIII and guinea pig liver transglutaminase [27]; (2) the region between residues 470–492, representing the highly charged region of protein 4.2 with a predicted α-helical structure; and (3) the ATP-binding P-type loop ^{346}Gly-Glu-Gly-Gln-Arg-Gly [21]. The requirement of the stretch of charged amino acids was suggested by the fact that E2 (which lacks the stretch of charged amino acids) is not capable of interacting with spectrin. Further recombinant proteins were analysed to obtain additional information about the spectrin-binding site. Since recombinant proteins G3 and G4 bound with equal affinity to spectrin, it was concluded that the stretch of amino acids beyond residue 492 was probably dispensable for spectrin binding. The binding affinities of G3 and H3 to spectrin were comparable, suggesting that amino acids upstream of residue 370 were also dispensable in the context of the spectrin-binding ability of protein 4.2. The hydrophobic stretch from amino acids 293–325 and the ATP-binding loop could therefore be excluded in defining the spectrin-binding site of protein 4.2. The inability of either recombinant proteins G2 or H2 to bind spectrin suggested that the region encompassing residues 470–492 is crucial in spectrin binding. This was confirmed further by the fact that peptide J3, encompassing residues 470–492 of protein 4.2, was able to associate with spectrin in solution and could also compete for the binding of ^{125}I-protein 4.2 to spectrin in solution. The K_d value of 0.70 μM obtained for binding with peptide J3 suggested that residues 470–492 are probably sufficient for the spectrin binding of protein 4.2. A search for similarity of this sequence performed using the BLAST algorithm [28] showed that residues 478–487 have significant sequence similarity (Figure 7) to a stretch of amino acids which form part of the middle α-helical domain of the ezrin, radixin, moesin ('ERM') family of proteins, key components of cytoskeletal structures, which regulate membrane dynamics and motility in eukaryotic cells [29]. It is tempting to hypothesize that these α-helical domains, common to cytoskeletal proteins of diverse origin, may interact with other α-helical domains of the same or different cytoskeletal proteins to stabilize cytoskeletal structures. It will be of interest to investigate this in greater detail.

The importance of the present study lies in the fact that this is the first report mapping the domain of protein 4.2 participating in its interaction with spectrin. Spectrin–protein 4.2 linkages are not indispensable for the maintenance of normal skeleton architecture. However, these interactions are likely to assume greater significance in situations where the membrane–skeleton interactions are compromised by deficiencies in other skeletal proteins, which are involved in maintaining vertical interactions between the skeleton and the bilayer.

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