Mapping of a palmitoylatable band 3-binding domain of human erythrocyte membrane protein 4.2

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Evidence accumulated over the years suggests that human erythrocyte membrane protein 4.2 is one of the proteins involved in strengthening the cytoskeleton–membrane interactions in the red blood cell. Deficiency of protein 4.2 is linked with a variety of hereditary haemolytic anaemia. 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 (CDB3). In order to carry out an initial characterization of its interaction with the CDB3, protein 4.2 was subjected to proteolytic cleavage and gel renaturation assay, and the 23-kDa N-terminal domain was found to interact with band 3. This domain contained two putative palmitoylatable cysteine residues, of which cysteine 203 was identified as the palmitoylatable cysteine. Recombinant glutathione S-transferase-fusion peptides derived from this domain were characterized with respect to their ability to interact with the CDB3. Whereas these studies do not rule out the involvement of other subsites on protein 4.2 in interaction with the CDB3, the evidence suggests that the region encompassing amino acid residues 187–211 is one of the domains critical for the protein 4.2–CDB3 interaction. This is also the first demonstration that palmitoylation serves as a positive modulator of this interaction.

Key words: anion exchanger, cytoskeleton, palmitoylation.

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 bi-concave shape of the erythrocyte, for its reversible deformability and for membrane structural integrity [1–3]. Spectrin tetramers interact with actin, protein 4.1 and adducin to form the cytoskeleton network [4–8]. This network is linked to the membrane through interactions between protein 4.1, p55 and glycophorin [9–12]; through ankyrin, which binds to the β-subunit of spectrin and attaches it to the membrane via band 3 [13,14]; through interactions between protein 4.2 and band 3; and through weak interactions of spectrin and protein 4.1 with the negatively charged lipids of the inner half of the bilayer [15,16]. A structural model has emerged that predicts two types of interaction: the vertical protein interactions ensure the anchoring of the skeleton to the lipid bilayer and comprise mainly the binding of band 3 to ankyrin, ankyrin to spectrin, glycophorin C to protein 4.1 and protein 4.2 to band 3. The horizontal protein interactions account for the lateral deformability and extensibility of the skeleton and consist mainly of spectrin dimer–dimer interactions and the spectrin–protein 4.1–actin-binding complex.

Protein 4.2 is a 72-kDa peripheral membrane protein that comprises approx. 5% of the total membrane protein [17–20]. It is present in 2×10^5 copies per red cell [21]. The importance of this protein has recently been recognized through studies that have shown that protein 4.2 deficiency results in overt haemolysis, anaemia and a borderline variety of hereditary spherocytosis [22–26]. The gene for protein 4.2 has been cloned and sequenced [27,28]. The protein shares significant sequence similarity with the transglutaminase family of enzymes and the gene for protein 4.2 is remarkably similar to the genes for human coagulation factor XIII and keratinocyte transglutaminase [29,30]. Protein 4.2 is neither a classical membrane skeletal nor an integral membrane protein. However, it promotes interactions between both these classes of proteins. Band 3–protein 4.2–ankyrin–spectrin interactions probably stablilize linkages between the cytoskeleton and the overlying membrane. Protein 4.2 deficiency associated with a borderline variety of hereditary spherocytosis may result either from a primary defect in protein 4.2 or from defects in some other proteins; primarily band 3, with which it associates. Protein 4.2 associates with band 3 *in vitro* [31], with ankyrin [31] and with spectrin [32]. Evidence from studies *in vivo* on ankyrin-deficient mouse red blood cells suggests that ankyrin enhances the association of protein 4.2 with the membrane [33]. Using protein 4.2-deficient erythrocyte membranes, it has been demonstrated that protein 4.2 controls the lateral [32] and the rotational [32,34] mobility of band-3 oligomers. Human erythrocyte protein 4.2 is N-terminally myristoylated [35] and palmitoylated [36]. 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 [37]. In addition, protein 4.2 shares a conserved 11-amino acid motif with dematin, which contains an ATP-binding P-loop [38]. Whereas it has been observed that the band 3–cytoskeleton linkage is weakened in protein 4.2-deficient membranes [39], there is an obvious gap in our knowledge at the molecular level of the interactions of protein 4.2 with band 3. In view of this, our objectives were to characterize the domains of protein 4.2

Abbreviations used: CDB3, cytoplasmic domain of band 3; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; TFA, trifluoroacetic acid; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, Nitro Blue Tetrazolium; IPTG, isopropyl thiogalactopyranoside; PAT, palmitoyl acyltransferase; PBS-T, PBS containing 0.5% Tween.

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involved in the interaction with the cytoplasmic domain of band 3 (CDB3) of the human erythrocyte, to identify the site(s) of palmitoylation of protein 4.2 and to find out the role, if any, of palmitoylation in regulating the protein 4.2–CDB3 interaction.

EXPERIMENTAL PROCEDURES

SDS/PAGE

Protein samples were separated on the basis of their molecular mass by SDS/PAGE according to the method of Laemmli [40]. SDS/PAGE using the discontinuous procedure of Schagger and von Jagow [41] was used for visualizing polypeptides of 2.5–17 kDa. Radiolabelled protein samples contained in SDS gels were visualized by fluorography according to the method of Bonner and Laskey [42].

Electroblotting on to PVDF and N-terminal sequencing

Electroblotting on to PVDF membranes was carried out as described by Matsudaira [43]. N-terminal sequencing was performed on an Applied Biosystems model 477A Pulsed Liquid Sequenator with online analysis of the phenylthiohydantoin amino acids by HPLC at the protein-sequencing facility of Eurosequence, Groningen, The Netherlands.

Preparation and radiolabelling of cells

Blood was collected from normal, healthy human volunteers in Dulbecco's modified Eagle's medium (DMEM). Cells were collected by centrifugation and washed with DMEM to remove the buffy coat. Packed erythrocytes (120 μl) were incubated at 37 °C with DMEM (8 ml) containing 20 μCi [9,10-^{3}H]palmitic acid (Amersham International) and 161 μg of fatty acid-free BSA. After 3 h, cells were pelleted, washed five times with DMEM containing 5 mg/ml BSA, twice with 155 mM NaCl/7.5 mM sodium phosphate/0.1 mM Na_2EDTA (pH 7.5) and lysed with lysis buffer (7.5 mM sodium phosphate, pH 7.5) containing 1 mM PMSF, 0.1 mM leupeptin and 30 μg/ml aprotinin. Ghosts were precipitated by centrifugation at 38000 ***g*** (Sorvall SS-34 rotor) for 20 min, followed by two washes in lysis buffer.

Purification and radio-iodination of protein 4.2

Human erythrocyte protein 4.2 was purified as described by Das et al. [36]. Protein 4.2 was radiolabelled with *N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate (Bolton–Hunter reagent) [44] and dialysed against 5 mM sodium phosphate/0.5 mM EGTA/0.5 mM dithiothreitol/120 mM KCl (pH 8).

Enzymic digestion of protein 4.2 with staphylococcal V8 protease

In order to dissect the site(s) of palmitoylation of protein 4.2, human erythrocytes were first labelled with [^{3}H]palmitic acid and peripheral protein extracts were prepared at pH 11 as described previously [36]. Protein 4.2 was electro-eluted from SDS gels of peripheral protein extracts at a concentration of approx. 150 μg/ml and partially digested with staphylococcal V8 protease (Pierce) [45], at an enzyme/protein ratio of 1:3 (w/w) for 3 h at 37 °C. The proteins in the reaction mixture were then precipitated by the addition of 5 vols. of chilled acetone, dried and loaded on to an SDS/polyacrylamide gel after treatment at 100 °C for 3 min with SDS-containing denaturing buffer.

CNBr digestion of the 23-kDa fragment of protein 4.2

Electroeluted protein 4.2 was digested partially with *Staphylococcus aureus* V8 protease as described above. The digest was run on an SDS/polyacrylamide gel (10% gel) and blotted on to a PVDF membrane. The blot was stained with Coomassie Brilliant Blue and the 23-kDa band was excised. The excised band was then treated with 200 μl of CNBr (10 mg/ml) in 70% formic acid for 24 h [46]. The digested extract was pipetted out and stored. Subsequently, the blot was extracted successively with 150 μl of 40% acetonitrile/0.1% trifluoroacetic acid (TFA) for 2 h at 37 °C, with 150 μl of 40% acetonitrile/0.1% TFA for 2 h at 50 °C and with 70% isopropanol/5% TFA for 2 h at room temperature with occasional vortexing. The extracts were pooled, dried, reconstituted in a minimum volume of distilled water and run on a 5–15% discontinuous gel according to the method of Schagger and von Jagow [41].

Purification of the CDB3 and biotinylation

Purification of CDB3 was performed according to Bennett [47]. Biotinylation was performed as described in the Pierce NHS-iminobiotin (*N*-hydroxysuccinimidoiminobiotin hydrobromide) instruction manual. Purified CDB3 (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 the biotinylated CDB3 was dialysed against the appropriate buffer.

Blot-overlay assay

This assay was performed as described by Platt et al. [48]. Protein 4.2 or recombinant protein 4.2-derived glutathione S-transferase

Table 1 Design of recombinant protein 4.2-derived peptides

Amino acid numbering is as according to the codon numbering of Sung et al. [28].

Peptide	Amino acids	5′ Primer	3′ Primer
C2	31–185	AAGGATCCATATGGGACAGGCACTGGGT	CGATGAATTCATGCGCTGAGCCTCATT
C3	187–260	CCGGATCCATGAGTACTTGTTGAAC	CGATGAATTCATGGTGGGCAGGAC
B1	100–211	CCGGATCCATTCCAAGATCAAC	ATGAATTCGCAAAGTCCCAGGACTC
R1	100–260	CCGGATCCATTCCAAGATCAAC	CGATGAATTCATGGTGGGCAGGAC
R2	100–185	CCGGATCCATTCCAAGATCAAC	CGATGAATTCATGCGCTGAGCCTCATT
R3	155–211	ATGGATCCATGGCAGGAAGCAACTC	ATGAATTCGCAAAGTCCCAGGACTC

(GST)-fusion proteins were electrophoresed on SDS/polyacrylamide gels. The proteins were transferred electrophoretically on to nitrocellulose paper. The blot was then blocked with blocking buffer [150 mM NaCl/10 mM sodium phosphate (pH 7.5)/0.05 % (v/v) Tween 20 containing 3 % (w/v) gelatin] for 30 min at room temperature. The blotted proteins were allowed to renature in renaturing buffer [150 mM NaCl/10 mM sodium phosphate (pH 7.5)/0.05 % (v/v) Tween 20/40 mg/ml BSA] for 1 h at room temperature. After thorough washing with ligand-blot buffer [150 mM NaCl/10 mM sodium phosphate (pH 7.5)/0.05 % (v/v) Tween 20], the blot was incubated with 1 μM biotinylated CDB3 for 1 h at room temperature. After the blot was washed with ligand buffer, it was incubated with 2 μg/ml streptavidin–alkaline phosphatase for 30 min at room temperature. After three washes, the colour was developed with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium (BCIP/NBT).

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 (a gift of Professor Carl Cohen, St. Elizabeth's Hospital, Boston, MA, U.S.A.) by PCR. The primers for the constructs are given in Table 1. The PCR-amplified products were purified, cloned in the vector pK19 [49], sequenced using the Sequenase Version 2.0 T7 DNA Sequencing Kit from United States Biochemicals, and finally transferred to the vector pGEX-3X (Pharmacia) 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 thiogalactopyranoside (IPTG) at 30 °C. After harvesting of 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 β-mercaptoethanol and 1 % (w/v) Triton X-100, and disrupted by sonication on a Labsonic 2000 sonicator (B. Braun) for a maximum of 10 s, avoiding frothing. 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 β-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 containing 126.6 mM NaCl, 10 mM sodium phosphate (pH 7.3), 5 mM β-mercaptoethanol, 5 mM EDTA, 20 % glycerol, 1 % 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/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.

Purification of the erythrocyte protein palmitoyl acyltransferase (PAT) and determination of the stoichiometry of palmitoylation by immunoprecipitation

Purification of the erythrocyte protein PAT was carried out as described by Das et al. [50]. The stoichiometry of palmitoylation of each of the recombinant proteins was also determined as described by Das et al. [50]. Briefly, varying amounts of the recombinant proteins (2–6 μg) were incubated with 40 μM [^{14}C]palmitoyl-CoA (50 mCi/mmol; Amersham International) and erythrocyte PAT (10 μg/ml) in 100 mM imidazole buffer (pH 7.5) for 60 min. Recombinant proteins were immunoprecipitated using anti-GST antibody and *Staph. aureus* Protein A, and the radioactivity associated with the bound antigen–antibody complex was counted in a liquid scintillation counter.

Binding assay for studying the interaction between protein 4.2-derived recombinant polypeptides and the CDB3

Where applicable, recombinant proteins were palmitoylated. Palmitoylation was checked by SDS/PAGE and fluorography. Proteins (40–80 μg in 100 μl) 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 blocking buffer [1 % (w/v) BSA in PBS-T] for 1 h at 37 °C. The wells were washed extensively with PBS-T. Biotinylated CDB3 [100 μl; 0.2–10 μM] was added to each well and the binding was allowed to proceed for 6 h at room temperature. The wells were then washed extensively with PBS-T. Binding of biotinylated CDB3 was measured by adding 0.1 μg/ml (in 100 μl) streptavidin–alkaline phosphatase to each well and incubating for 60 min at room temperature. 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.

The inhibition of the association of protein 4.2 with biotinylated CDB3 by recombinant fusion protein was studied by allowing [^{125}I]protein 4.2 (18–72 μg/ml) to interact with biotinylated CDB3 (40 μg) in a 100-μl vol. of 120 mM KCl/5 mM sodium phosphate/0.1 mM EGTA/20 μg/ml PMSF/0.5 mM dithiothreitol/1 mg/ml gelatin (pH 8). This was at room temperature in the absence or presence of different concentrations of palmitoylated or non-palmitoylated recombinant fusion protein. Streptavidin–agarose (10 μl of a 50 % slurry) was then added to each tube, followed by incubation for 30 min at room temperature. The suspension was centrifuged at 2000 ***g*** in a clinical centrifuge, the streptavidin–agarose beads were washed twice with 100 μl of binding buffer, and the pellet was counted in a γ-counter. Control experiments lacking biotinylated CDB3 were run in parallel to account for non-specific binding.

RESULTS

Partial proteolysis of labelled protein 4.2 and blot-overlay assay

Electroeluted protein 4.2 was proteolysed partially with staphylococcal V8 protease and loaded in three separate lanes of a denaturing SDS/polyacrylamide gel. After running the gel, one lane was stained with Coomassie Brilliant Blue and subjected to fluorography (in order to identify the site of palmitoylation), the proteins of a second lane were transferred on to a nitrocellulose membrane (for blot-overlay assay) and the proteins of a third lane were transferred on to a PVDF membrane (for N-terminal sequencing).

The proteins transferred on to nitrocellulose were renatured as described. After incubation with blocking buffer in order to block non-specific binding, the renatured proteins were incubated with biotinylated CDB3 followed by successive incubations with streptavidin–alkaline phosphatase and a mixture of BCIP/NBT. It was observed that a 23-kDa band developed colour (Figure 1A, lane b). This 23-kDa polypeptide was therefore likely to

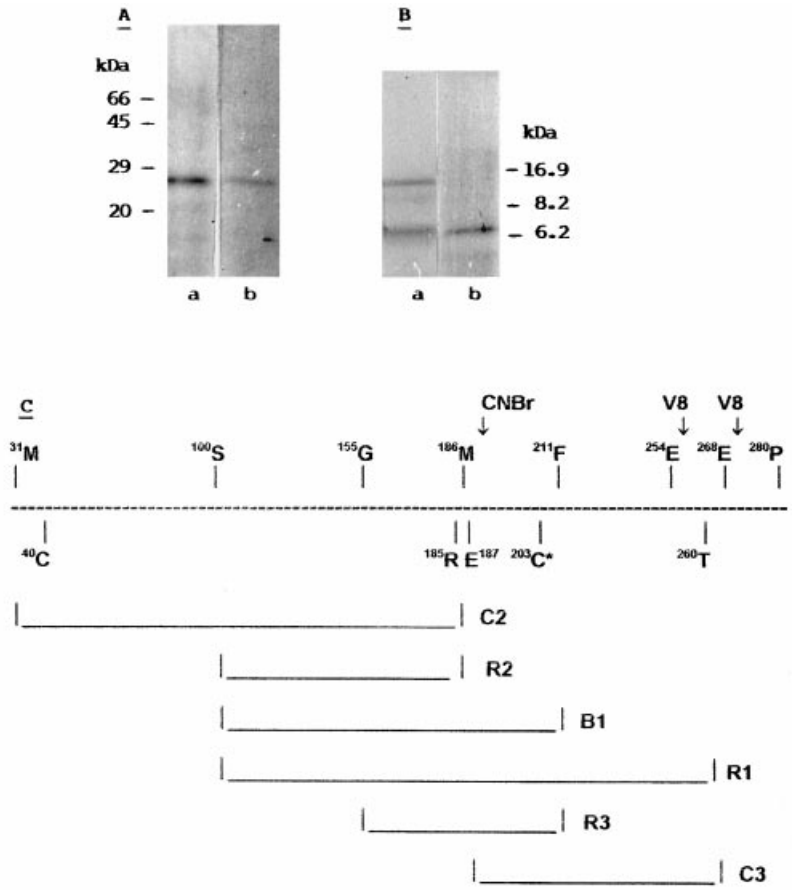

Figure 1 Analysis of the CDB3-interacting domain and the site(s) of palmitoylation of protein 4.2

(**A**) Partial proteolysis of protein 4.2 with V8 protease, analysis of the interaction of the proteolytically derived peptides with the CDB3 and identification of the palmitoylated peptide(s). The [^{3}H]palmitate-labelled proteolytically derived peptides were run on SDS/polyacrylamide gels followed by fluorography (lane a); or transferred on to nitrocellulose, renatured and incubated with the biotinylated CDB3, followed by colour development with streptavidin–alkaline phosphatase and BCIP/NBT (lane b). (**B**) Cleavage of the [^{3}H]palmitate-labelled 23-kDa polypeptide with CNBr and identification of the palmitoylated peptide(s). Lane a, Coomassie Brilliant Blue-stained Schagger and von Jaggow [41] gel of the peptides obtained after CNBr treatment. Lane b, fluorography of the same gel. (**C**) Map of the N-terminal ($\approx$ 23-kDa) domain of protein 4.2 and the recombinant polypeptides indicating the sites of cleavage of V8 protease and CNBr. ^{203}C* represents the putative palmitoylatable cysteine residue. The positions of molecular-mass markers have been indicated. Amino acid numbering is according to that of Sung et al. [28].

represent the band-3-interacting domain. A 23-kDa polypeptide also gave a band after fluorography (Figure 1A, lane a), indicating that it was palmitoylated. No other band appeared after fluorography.

N-terminal sequencing

The 23-kDa polypeptide blotted on to a PVDF membrane was subjected to N-terminal sequencing and found to be N-terminally blocked. This suggested that the 23-kDa polypeptide represented the N-terminal domain of protein 4.2, since the N-terminus of protein 4.2 is blocked by N-myristoylation. The different N-terminal domains of protein 4.2 referred to subsequently are represented in Figure 1(C). The 23-kDa polypeptide was subjected to cleavage by CNBr, taking note of the fact that the predicted domain contained only one methionine residue at position 186. CNBr treatment would cleave this domain into polypeptides of approx. 16000 (containing one putative cysteine residue at position 40) and 6500 Da (containing one putative cysteine residue at position 203). Treatment of the [^{3}H]palmitate-labelled 23-kDa fragment with CNBr, followed by SDS/PAGE and fluorography, showed that only one 6.5-kDa peptide was labelled (Figure 1B). Protein 4.2 therefore appeared to contain only one palmitoylatable cysteine at position 203. N-terminal sequencing showed that, as predicted, the 16-kDa peptide was N-terminally blocked and the N-terminal sequence of the 6.5-kDa peptide was ^{187}Glu-Tyr-Leu-Leu-Asn-Gln-Asn-Gly-Leu-Ile-Tyr^{197}.

In connection with the above experiments, in particular partial proteolysis with V8 protease, it is pertinent to mention that molecular-mass determination by SDS/PAGE is approximate. The more accurate methods of mass spectrometry could not be used here, since these would have been hampered by the presence of SDS (V8 protease digestion having been performed in the presence of SDS). However, the span of the 23-kDa domain was determined after taking several points into careful consideration. Cleavage at Glu-254 would generate a fragment of 22.6 kDa, whereas cleavage at Glu-268 would yield a fragment of 24.2 kDa. In the first instance, subsequent CNBr cleavage would yield fragments of 16 and 6.6 kDa respectively. In the second instance, fragments of 16 and 8.2 kDa respectively would be obtained. For practical purposes it was considered sufficient to consider the molecular masses of the amino acids only and exclude the post-translational modifications such as myrist-

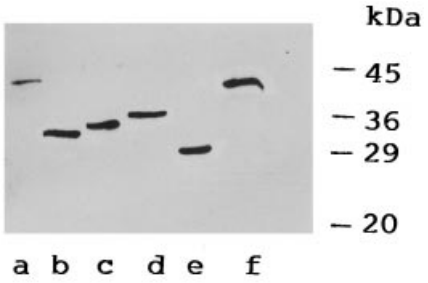

Figure 2 Purification of the recombinant fusion proteins

Coomassie Brilliant Blue-stained SDS/polyacrylamide gels of the fusion proteins purified on glutathione–Sepharose 4B. Lanes a–f represent the proteins C2, C3, R2, B1, R3 and R1 respectively. The positions of molecular-mass markers have been indicated.

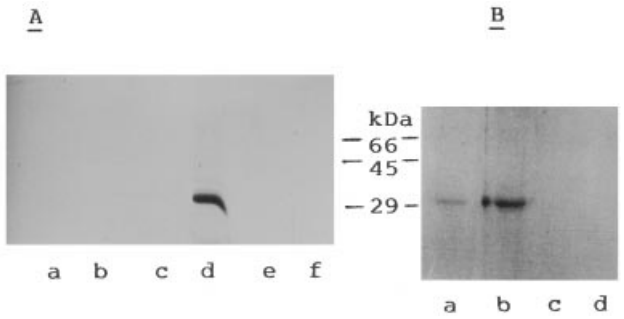

Figure 3 Palmitoylation of recombinant GST-fusion proteins C2 and C3 and blot-overlay assay of the interaction of C3 with biotinylated CDB3

(**A**) Fusion proteins (3 μg) were palmitoylated in the absence (lanes a, c and e) or presence (lanes b, d and f) of the erythrocyte PAT and [^{14}C]palmitoyl-CoA, followed by fluorography. Lanes a and b, recombinant GST; lanes c and d, C3; and lanes e and f, C2. (**B**) Purified fusion protein (3 μg in each lane) was run on an SDS/polyacrylamide gel and subjected to blot-overlay assay using biotinylated CDB3 as described. Lane a, non-palmitoylated C3; lane b, palmitoylated C3; lane c, C2; and lane d, recombinant GST. The positions of molecular-mass markers have been indicated.

oylation and palmitoylation. Since Shagger and von Jagow [41] gels that had been run with appropriate molecular-mass markers showed the presence of polypeptides of 16 and 6.5 kDa, it was assumed that the N-terminal V8 protease-generated polypeptide terminated at Lys-253.

Expression of recombinant GST-fusion proteins

Expression of fusion proteins in *E. coli* was chosen as a strategy for studying the binding of these domains with the CDB3. The rationale was that the fusion proteins expressed in *E. coli* would represent the non-palmitoylated state, since palmitoylation does not occur in the prokaryote, and that these fusion proteins could be palmitoylated subsequently by the erythrocyte PAT and the binding with CDB3 assessed.

Expression of fusion proteins spanning amino acid residues 31–185 (C2) and 187–260 (C3) was carried out by induction with 0.1 mM IPTG at 30 °C and fusion proteins of the desired size (i.e. approx. 33 and 42 kDa for C3 and C2 respectively) were expressed. The expression of C2 was not to the level of that observed for C3, and some amount of a band corresponding to GST was always observed. The harvested cells were sonicated with a brief pulse. After centrifugation, the supernatant and the pellet were analysed by SDS/PAGE. The majority of the fusion proteins were found in the post-sonicate pellet. Decreasing the temperature used for induction with IPTG, or the concentration of IPTG, failed to produce better results. Solubilization was achieved with urea, the urea extract was allowed to adsorb to glutathione–Sepharose 4B and elution was carried out with glutathione. The presence of 0.1 % Triton X-100 in the elution buffer was found to be essential for efficient elution of the fusion protein. C3 was purified to > 90 % purity (Figure 2). Efforts to purify C2 yielded, at best, low amounts of this fusion protein from the glutathione–Sepharose 4B resin. Attempts to express and purify fusion proteins encompassing amino acid residues 31–260 (i.e. the N-terminal 23-kDa domain) as well as full-length protein 4.2 were not successful.

Palmitoylation of fusion proteins with the erythrocyte PAT

The purified fusion protein (3 μg) was palmitoylated with 40 μM [^{14}C]palmitoyl CoA in the absence or in the presence of erythrocyte PAT, and run on an SDS/polyacrylamide gel, followed by fluorography. From Figure 3(A) it is evident that the palmitoylation of C3 was mediated by the erythrocyte PAT. No palmitoylation was observed in the absence of the enzyme. C2 could not be palmitoylated. Recombinant GST was run as a control after palmitoylation. No band appeared after fluorography, indicating that the band observed with C3 reflected S-acylation of the palmitoylatable cysteine of protein 4.2. Incorporation of the palmitoyl moiety into C3 occurred in a molar ratio of 1:1.

Blot-overlay assay

The palmitoylated C3 was obtained by acylation of the purified protein in the presence of erythrocyte PAT, followed by purification on glutathione–Sepharose 4B. A blot-overlay assay with recombinant fusion proteins (3 μg) and biotinylated CDB3 was performed. No band was obtained corresponding to C2 (Figure 3B). This experiment therefore could not demonstrate interaction between the 16-kDa N-terminal domain of protein 4.2 and CDB3. Positive bands were obtained with C3 in its palmitoylated and non-palmitoylated form (Figure 3B). However, the intensity of the band was more in the case of the palmitoylated fusion protein. This suggested that palmitoylation could modulate positively the interaction of the fusion protein with the CDB3. No colour development was observed in the control run with the recombinant GST alone, suggesting that non-specific interactions between GST and the CDB3 were not responsible for the colour development observed with the recombinant fusion protein. Since, from the blot-overlay assay, C3 and not C2 appeared to interact with the CDB3, only the interaction of C3 with the CDB3 was studied further.

Interaction of C3 with CDB3

The interaction of C3 with CDB3 was studied by first allowing the recombinant fusion proteins to bind to microtitre plates, followed by successive incubations with biotinylated CDB3, streptavidin–alkaline phosphatase and *p*-nitrophenyl phosphate. The A_{405} values obtained were converted to micrograms of CDB3 bound by preparing a standard curve of A_{405} versus [biotinylated CDB3]. In each experiment, recombinant GST alone was run as a control. It did not interact with the CDB3. Non-specific binding (i.e. readings obtained for BSA or GST) was less than 15 %. It was observed that C3 interacted with the CDB3. Five experiments were performed using different preparations of the CDB3 and recombinant fusion proteins. The

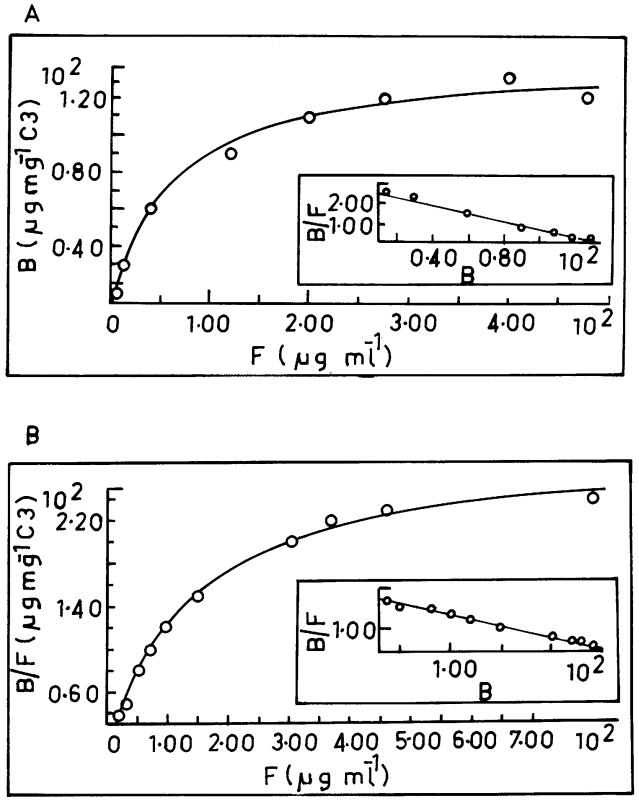

Figure 4 Scatchard analysis of the interaction of C3 with the CDB3

The binding of palmitoylated (**A**) and non-palmitoylated (**B**) fusion proteins with biotinylated CDB3 were assayed in microtitre plates. Data were analysed using the non-linear-regression curve-fitting software ENZFITTER (Elsevier Biosoft).

Scatchard plot appeared linear (Figure 4), suggesting a single class of sites interacting with the CDB3. The K_d values of the non-palmitoylated form ranged from 1.7 to 3.6 μM, whereas the K_d values of the palmitoylated form ranged from 0.6 to 1.8 μM. Variations in K_d values similar to those obtained during this study have been reported by Korsgren and Cohen [31]. Figure 4 shows a representative set of data of the interaction of the CDB3 with the palmitoylated and the non-palmitoylated forms of the fusion protein. A pairwise comparison of data for the palmitoylated and the non-palmitoylated fusion proteins from each of the five sets of experiments showed at least a 3-fold decreased K_d value for the palmitoylated form, suggesting that palmitoylation enhances the affinity of the fusion protein for the CDB3. The K_d values suggest that it is likely to represent an important domain of protein 4.2 involved in interaction with the CDB3.

Inhibition of the interaction of [^{125}I]protein 4.2 with the CDB3 by C3

In conformity with the previous observations, it was found that the interaction of [^{125}I]protein 4.2 with the CDB3 could be inhibited by C3 (Figure 5). Recombinant GST alone could not inhibit the interaction (data not shown). Non-specific binding was less than 15%. Palmitoylation increased the inhibitory ability of the fusion protein. No further inhibition was observed with increasing concentrations of recombinant fusion protein.

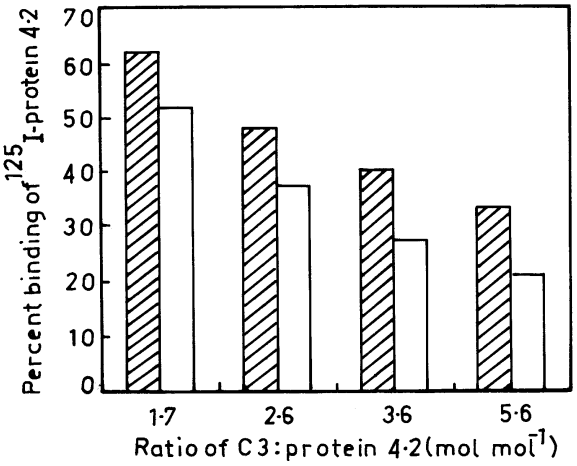

Figure 5 Competitive inhibition of protein 4.2 binding to the CDB3 by C3

[^{125}I]Protein 4.2 (60 μg/ml) was allowed to react with biotinylated CDB3 (40 μg) in the absence or presence of various concentrations of C3. The binding of protein 4.2 was determined after precipitation of the protein 4.2–CDB3 complex with streptavidin–agarose. The binding of protein 4.2 in the absence of C3 was taken to be 100% and the percentage binding was calculated relative to this. The binding of protein 4.2 in the presence of non-palmitoylated and palmitoylated C3 is shown by hatched bars and white bars respectively. Data represent the means of three separate determinations, with each S.D. $\leqslant$ 5% of the mean.

Table 2 K_d values for binding of recombinant protein 4.2-derived peptides with the CDB3

Results represent the mean of five separate determinations. Figures in parentheses represent the range of values obtained. NA, not applicable; these recombinants did not contain the palmitoylable cysteine. ND, not determined; the binding was too low, and reliable estimates of K_d were not possible.

	K_d (μM)	
Recombinant protein	Palmitoylated	Non-palmitoylated
C3	0.8 (0.6–1.2)	2.9 (1.7–3.6)
B1	0.9 (0.7–1.3)	3.2 (2.0–4.0)
R1	0.8 (0.6–1.3)	3.0 (1.9–3.7)
R2	NA	ND
R3	NA	ND

Expression and purification of recombinant proteins B1, R1, R2 and R3, and interaction with the CDB3

Several mutations have been identified upstream of amino acid residue 187 in cases of hereditary haemolytic anaemias with protein 4.2 deficiency, such as the cases of protein 4.2 Nippon (Ala^{142} $\rightarrow$ Thr) and protein 4.2 Komatsu (Asp^{175} $\rightarrow$ Tyr) [51,52]. In view of this it was thought to be relevant to analyse whether recombinant proteins encompassing these residues were more efficient in binding to the CDB3 than the recombinant C3. In an effort to map the minimal domain of protein 4.2 involved in binding to the CDB3, several more recombinant proteins were expressed (Table 1), purified as described in the case of C3 (Figure 2), and their binding to the CDB3 was studied using the assay described. Recombinants carrying the palmitoylatable cysteine were palmitoylated (in a molar ratio of 1:1 of fatty acid to protein). Table 2 summarizes the results obtained. R2, which encompassed amino acid residues 100–185, was unable to bind to the CDB3. R1, encompassing amino acid residues 100–260, C3,

encompassing residues 187–260, and B1, encompassing residues 100–211, showed comparable binding affinities. Residues 187–211, encompassing the palmitoylatable cysteine, therefore appeared to define a crucial CDB3-binding domain of protein 4.2.

DISCUSSION

Protein 4.2 represents 5% of the total membrane proteins of the human erythrocyte, and non-erythroid analogues are present in diverse cell types. However, the major interactions of human erythrocyte protein 4.2 with other proteins of the erythrocyte membrane, particularly with band 3, remain to be characterized at the molecular level. This study has attempted to map, at least in part, the domain of protein 4.2 involved in binding with the CDB3. Based on our previous observations that protein 4.2 palmitoylation modulates its binding to membrane vesicles [36], we have also attempted to delineate the role, if any, of palmitoylation in modulating CDB3–protein 4.2 interactions, since band 3 represents the high-affinity binding site of protein 4.2 on the membrane. Partial proteolysis of protein 4.2, followed by a blot-overlay assay, identified the N-terminal 23-kDa domain of protein 4.2 as the band-3-interacting domain. While this work was in progress, Rybicki et al. [34] also demonstrated that the N-terminus of protein 4.2 encompasses the band-3-binding site. This domain contains two putative cysteine residues. Either or both of these residues could potentially be palmitoylated. CNBr cleavage of the [^{3}H]palmitate-labelled 23-kDa polypeptide showed that only the 6.5-kDa peptide gave a band on fluorography. It therefore appeared that the palmitoylatable cysteine residue was located at position 203. Using synthetic peptides, Rybicki et al. [34] had defined the CDB3 binding site as being contained within a stretch encompassing residues 63–75. However, our subsequent experiments differed from these observations. Blot-overlay experiments with the N-terminal 16-kDa-expressing fusion protein could not demonstrate its interaction with the CDB3. On the other hand, the fusion protein expressing the domain Glu-187–Thr-260 interacted with the CDB3. The interaction was saturable, and the Scatchard analysis showed the presence of a single class of binding site, corroborating the observations of Korsgren and Cohen [31] using intact protein 4.2. Scatchard analysis of the binding of the peptide identified by Rybicki et al. had reported the presence of both high- and low-affinity binding sites. Palmitoylation was found to act as a positive modulator of this interaction. Since the 16-kDa-domain-expressing fusion protein could not be purified in substantial amounts, quantitative analysis of its interaction with the CDB3 could not be carried out. The significance, if any, of the domain identified by Rybicki et al. [34] could therefore not be ruled out. However, the use of short synthetic peptides for such studies has inherent pitfalls. The recombinant protein C3 showed a moderate affinity for interaction with the CDB3. Whether this domain was optimal for the interaction was further investigated, considering the fact that several sites of protein 4.2 mutations linked with hereditary spherocytosis, and a total to partial absence of protein 4.2 from the membrane, reside outside this domain. It was observed that the critical region for interaction with the CDB3 was that encompassed by residues 187–211. Residues 142 (mutated in protein 4.2 Nippon) or 175 (mutated in protein 4.2 Komatsu) are therefore probably not involved directly in the CDB3-binding site, but mutations at these sites probably impact the tertiary structure of protein 4.2 enough to affect the CDB3-binding site. The difference in K_d values between the recombinant fusion proteins described here and that reported by Korsgren and Cohen using intact protein 4.2 [31] could be due to several reasons. One of these could be the use of different binding-assay systems. As an example, the dystrophin actin-binding affinity has been reported to range from 0.1 [53] to 44 μM [54]. On the other hand, the differences in K_d values could also reflect the possibility that other sites in protein 4.2 may also participate in, or contribute to, CDB3 binding. This possibility is also reflected by the fact that complete inhibition of the binding of protein 4.2 with the CDB3 could not be achieved using recombinant C3 (Figure 5).

Recent studies have demonstrated elegantly, in several instances, the ability of palmitoylation to modulate protein–protein interactions. Such effects are likely to be mediated by subtle local changes in protein conformation induced by protein palmitoylation. Recently, palmitoylation of the α subunit of the guanine nucleotide-binding protein G_z has been shown to inhibit its response to the GTPase-accelerating activity of G_z GTPase-activating protein, a member of the regulators of G-protein signalling protein family of GTPase-activating proteins [55]. In our studies, palmitoylation was found to enhance the affinity of the proteins C3, B1 and R1 for the CDB3. The effect was observed consistently, suggesting that it was likely to bear a significance in modulating the vertical interactions between the erythrocyte membrane and the cytoskeleton in a manner which remains to be understood fully. The fact that depalmitoylation by hydroxylamine treatment of native protein 4.2 resulted in a more pronounced effect in terms of the binding of protein 4.2 to the membrane [36] than evidenced in our experiments with the recombinant proteins could be due to either of two reasons. Hydroxylamine could possibly alter the native conformation of the protein. Alternatively, palmitoylation of protein 4.2 could, in addition to bringing about local changes in protein 4.2 conformation, also result in additional associations of the palmitate moiety with the hydrophobic components of the membrane, resulting in increased affinity of protein 4.2 for the membrane, a factor which does not arise when studying protein 4.2–CDB3 interactions. The effects of protein 4.2 palmitoylation are likely to be complex and multifaceted. The importance of the present study rests in the fact that not only is it one of the first documented reports on mapping the domain of protein 4.2 participating in interaction with the CDB3, but that it also provides, for the first time, insights into the potential role of palmitoylation in modulating protein 4.2–membrane interactions.

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