Identification of a band-3 binding site near the N-terminus of erythrocyte membrane protein 4.2

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Protein 4.2 (P4.2) is a major component of the erythrocyte plasma membrane accounting for approx. 5% of total membrane protein. The major membrane binding site for P4.2 is contained within the cytoplasmic domain of band 3 (cdb3), although the precise location of the cdb3 binding site is not known. To identify the cdb3 binding site, we used synthetic P4.2 peptides (15-mers) that spanned the entire 721-amino-acid large isoform of P4.2, and determined the binding of these peptides to cdb3 in an *in vitro* binding assay. One peptide, P8 (L⁶¹FVRRGQPFTIILYF), bound strongly to cdb3 and four others bound less strongly (P22, L²⁷¹LNKRRGSPILRQW; P27, G³⁴⁶EGQRGRIWIFQTST; P41, L⁵⁵⁶WRKKLHLTL-SANLE; P48, I⁶⁶¹HRERSYFRFRSVWPE). These peptides have in common a cluster of two or three basic amino acid residues (arginine or lysine), in a region without nearby acidic residues.

Cdb3 bound saturably to P8 with a K_d of 0.16 μ M and a capacity of 0.56 mol of cdb3 monomer/mol of P8. Use of overlapping synthetic peptides further defined the cdb3 site as being contained within V⁶³RRGQPFTIILYF. Replacement of R⁶⁴R with R⁶⁴G, G⁶⁴R or G⁶⁴G almost completely abolished cdb3 binding, suggesting that R⁶⁴R is essential for cdb3 binding. P8 competitively inhibited binding of purified human erythrocyte P4.2 to cdb3. In blot overlay assays, cdb3 bound to a 23 kDa N-terminal P4.2 tryptic peptide containing V⁶³RRGQPFTIILYF but not to other P4.2 tryptic peptides lacking this site. The V⁶³RRGQPFTIILYF site is highly conserved in mouse and human erythrocyte P4.2 as well as between P4.2 and transglutaminase proteins, which are evolutionarily related to P4.2.

INTRODUCTION

Erythrocyte protein 4.2 (P4.2), a 72 kDa peripheral membrane protein, is a major component of the human erythrocyte membrane, the function of which remains unknown. Studies on a group of Japanese patients with haemolytic anaemia whose erythrocytes are abnormally shaped, osmotically fragile and more than 99% deficient in P4.2 suggests that P4.2 is required to stabilize erythrocyte membrane integrity [1,2]. The mutation in P4.2 in these patients has been identified (P4.2^{NIPPON}) and is strongly associated with the P4.2 deficiency [2]. Studies on mice carrying the pigmentation mutation *pallid* (*pa/pa*) suggest that the *pallid* phenotype may be caused by an abnormal P4.2 gene [3]. The *pallid* mutation is associated with albinism and platelet-storage-pool deficiency and is considered to be a model for defects of human platelet dense granules [4], suggesting the possibility that non-erythroid forms of P4.2 may have important functions in melanogenesis and coagulation.

Human erythrocyte P4.2 binds strongly to the membrane, as evidenced by the harsh conditions required to dissociate it, such as strongly alkaline pH (pH > 11) and chaotropic salt or detergent denaturation [5]. Early work by Yu and Steck [6] suggested that the membrane binding site for erythrocyte P4.2 was band 3, which was later confirmed by Korsgren and Cohen [7], who further showed that purified P4.2 associated strongly with the cytoplasmic domain of band 3 (cdb3) and suggested that cdb3 represented the major membrane attachment site for P4.2 in human erythrocytes. In addition to providing a binding site for P4.2, cdb3 also contains binding sites for several other membrane and cytosolic proteins including ankyrin, protein 4.1, haemoglobin and glycolytic enzymes [8]. The location of the cdb3 binding site in P4.2 is not known, which is in part due to

technical difficulties in purifying functional P4.2 fragments. Our initial approach to locating the cdb3 binding site was to use a series of synthetic P4.2 peptides that spanned the entire predicted 721-amino-acid sequence of human erythrocyte P4.2 [9]. The peptides were biotinylated (with four-residue spacers) to increase their solubility and to allow their detection *in vitro* in binding assays with cdb3. Using this approach we have identified a major cdb3 binding site contained within V⁶³RRGQPFTIILYF.

EXPERIMENTAL

Materials

Avidin and biotin were from Zymed (South San Francisco, CA, U.S.A.). Streptavidin, BSA, ovalbumin, Tween 20, PMSF, *p*-nitrophenyl phosphate, chymotrypsin and chymotrypsin inhibitor came from Sigma (St. Louis, MO, U.S.A.). Flat-bottomed 96-well enzyme immunoassay (EIA) microtitre plates (type 3590) were purchased from Costar (Cambridge, MA, U.S.A.). NHS-LC biotin and 2-(4'-hydroxyazobenzene)benzoic acid reagent were obtained from Pierce (Rockford, IL, U.S.A.). Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (H+L) and goat anti-rabbit IgG (H+L) antibodies were from Bio-Rad (Richmond, CA, U.S.A.).

Peptide and protein purification and handling, and primary antibodies used

P4.2 peptides [biotinylated 15-mers with a four-amino-acid (SGSG) N-terminal spacer] were synthesized by Chiron Mimotopes. The peptides spanned the entire predicted amino acid sequence of the large isoform (721-amino-acid isoform I) of

Abbreviations used: P4.2, erythrocyte-protein 4.2; cdb3, cytoplasmic domain of band 3; EIA, enzyme immunoassay; AP, alkaline phosphatase.

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Table 1 Primary antibodies used in binding studies *in vitro*

Antibody	Description	Reference
Anti-P4.2 72 kDa	Rabbit polyclonal antibody derived from purified human erythrocyte P4.2. Recognizes epitopes throughout P4.2	[1]
Anti-P4.2-(96–105)	Rabbit polyclonal antibody derived from P4.2 synthetic peptide E ¹²⁶ RDAQSWTIS (numbering as described in Sung et al. [9]); note that in the numbering system of Korsgren et al. [11] this peptide would begin at residue 96). Recognizes epitope near N-terminus of human erythrocyte P4.2. Conjugation of this peptide to keyhole limpet haemocyanin was identical with that described in ref. [4]	
Anti-P4.2-(479–488)	Rabbit polyclonal antibody derived from P4.2 synthetic peptide E ⁴⁷⁹ RVEKEKMER (numbering as described by Sung et al. [9]). Recognizes epitope near C-terminus of human erythrocyte P4.2	[2] (referred to as anti-P2)
Anti-cdb3	Mouse monoclonal antibody that recognizes N-terminus of human erythrocyte cdb3 (kindly provided by Dr. P. Büttikofer [13]). Similar antibody described by Czerwinski et al. [12]	[13]

human erythrocyte P4.2 (numbering as described in ref. [9]). The peptides synthesized were more than 70% pure, as verified by reverse-phase HPLC or MS. Lyophilized peptides were resuspended at 1–2 mg/ml in 30% acetonitrile and sonicated for 30 min or until dissolved. Most of the peptides were dissolved completely by this procedure, but some very hydrophobic ones remained partially undissolved in which case additional acetonitrile was titrated until the peptide was completely dissolved. Peptides in solution were divided into portions and stored at –70 °C. Human erythrocyte cdb3, a water-soluble 40 kDa chymotryptic fragment of band 3, was prepared by the method of Bennett [10]. Human erythrocyte P4.2 was prepared by the method of Korsgren and Cohen [7]. Primary antibodies used to detect P4.2 and cdb3 are described in Table 1. Protein concentrations were determined by the Bio-Rad protein assay with bovine γ -globulin as standard or by the Pierce bicinchoninic acid protein assay with BSA as standard.

Binding of synthetic P4.2 peptides to cdb3

Protein-binding assays were performed as suggested by Chiron Mimotopes [14]. Briefly, 96-well EIA microtitre plates were incubated overnight at 37 °C with 500 ng of streptavidin/well. The plates were then washed five times with PBS containing 0.1% Tween 20 (PBS/Tween). Non-specific protein-binding sites were blocked by incubating the plates for 2 h at 25 °C with 2% BSA in PBS and then washing in PBS/Tween as above. The plates were then incubated with 5 μ g of each biotinylated peptide in PBS containing 0.1% BSA and 0.1% NaN₃ for 1 h at 25 °C on a microtitre-plate shaker. Peptide dilutions were from sonicated (20–30 min) peptide stocks. After incubation, the plates were washed as above and incubated for 2 h at 25 °C or 18 h at 4 °C with 25–50 μ g of cdb3 in cdb3–P4.2-binding buffer (120 mM KCl, 5 mM sodium phosphate, pH 8.0, 0.5 mM EGTA, 0.5 mM dithiothreitol, 0.1% BSA, 0.02% NaN₃). For some experiments, cdb3 was iodinated using Bolton–Hunter ¹²⁵I reagent (Amersham). The wells were then washed five times in

PBS/Tween. Bound cdb3 was detected either by counting radioactivity in each well (when radiolabelled cdb3 was used) or immunologically with anti-cdb3 antibody (see Table 1) (1:500 dilution) followed by AP-conjugated goat anti-mouse antibody (1:250 dilution). Colour was developed with AP substrate (*p*-nitrophenyl phosphate) for 1 h at 25 °C. The reaction was terminated by the addition of 0.1 M EDTA and the A_{405} was recorded using a microtitre plate reader (TiterTek).

Inhibition of P4.2 binding to cdb3 by P4.2 peptides

Cdb3 (2 mg) was biotinylated using NHS-LC biotin according to the manufacturer's instructions. Biotinylated cdb3 (5 μ g; 0.13 nmol) was bound to streptavidin-coated 96-well EIA microtitre plates as above. After binding, unchanged streptavidin sites were blocked by addition of excess biotin. This was followed by the simultaneous addition of purified human erythrocyte P4.2 and synthetic P4.2 peptides at molar ratios of 0.2–55 mol of peptide/mol of P4.2. The mixture was incubated for 18 h at 4 °C, as described by Korsgren and Cohen [7]. Bound P4.2 was detected by incubation for 2 h at 25 °C with anti-P4.2-(479–488) (see Table 1) (1:40 dilution), followed by AP-conjugated goat anti-rabbit secondary antibody (1:500 dilution) and AP substrate as above. The anti-P4.2-(479–488) antibody recognizes human erythrocyte P4.2 but not the synthetic P4.2 peptides tested (P8, P22, P27, P41, P48).

Binding of cdb3 to P4.2 tryptic peptides

P4.2, purified from human erythrocyte membranes as described above, was subjected to limited treatment with trypsin at a ratio of P4.2/trypsin of 50:1 (w/w), as described by Dotimas et al. [15]. The peptides were separated by SDS/PAGE (12% acrylamide) using a Laemmli buffer system [16] and transferred to nitrocellulose [17]. The nitrocellulose was incubated with 3% gelatine in 10 mM Tris/HCl (pH 7.4)/140 mM NaCl (TBS) for 2 h at 25 °C to block non-specific protein-adsorption sites, washed in TBS, air-dried and cut into strips. Some strips were incubated with cdb3 (> 250 μ g/ml) in P4.2–cdb3 binding buffer for 18 h at 25 °C. After being washed (five times in TBS) to remove unbound cdb3, bound cdb3 was detected using anti-cdb3 antibody (see Table 1) (1:500 dilution) followed by AP-conjugated goat anti-mouse IgG (H + L) (1:1333 dilution) and AP colour reagents. To visualize the P4.2 tryptic peptides, other nitrocellulose strips were incubated with anti-P4.2 72 kDa antibody (see Table 1) (1:40 dilution) followed by AP-conjugated goat anti-rabbit IgG (H + L), as above.

RESULTS

Cdb3 binding to synthetic P4.2 peptides

Addition of human erythrocyte cdb3 to immobilized biotinylated synthetic P4.2 peptides resulted in specific binding to five peptides (specific binding is defined as binding at more than 1 S.D. above the mean for all 48 peptides). The five peptides were P8, P22, P27, P41 and P48, with the greatest binding to P8 (Figure 1). [Note that the synthetic peptides are numbered with P4 containing the P4.2 N-terminus (P1, P2 and P3 are synthesis-control peptides)]. Analysis of these five peptides revealed a common motif that included the presence of two or more basic amino acids (RR bound more strongly than RK or KK) in a domain that does not contain nearby acidic residues. Cdb3 bound saturably to P8 with a K_d of 0.16 μ M and a predicted maximum capacity of 0.56 mol

Figure 1 Cdb3 binding *in vitro* to synthetic P4.2 peptides

Some 48 P4.2 peptides (15-mers) spanning the entire sequence of the large isoform of human erythrocyte P4.2 were synthesized (note that the N-terminal peptide is numbered P4). These peptides were biotinylated at the N-terminus with four-residue (SGSG) spacers and immobilized on streptavidin-coated 96-well EIA microtitre plates. Cdb3 was purified from human erythrocytes and binding to the immobilized peptides was as described in the Experimental section. Specific binding of cdb3 to the P4.2 peptides was defined as binding > 1 S.D. above the mean for all 48 peptides. Five P4.2 peptides (P8, P22, P27, P41, P48) met this binding criterion, and their locations in P4.2 are indicated. These five P4.2 peptides shared clusters of basic amino acids (Indicated by bold letters) in a domain free of acidic amino acids.

Figure 2 Binding isotherm for cdb3 binding to synthetic P4.2 peptide P8

The binding assay is similar to that in Figure 1 except that cdb3 was iodinated and binding was quantified by counting bound radioactivity. The binding curve was generated using Enzfitter non-linear regression curve-fitting software (version 1.04; Biosoft, Cambridge, U.K.).

of cdb3 monomer/mol of P8 (Figure 2). Scatchard analysis of this binding isotherm revealed both high-affinity and low-affinity cdb3 binding sites (Figure 2).

9 R76APVRAFLPALKKVA
8 L61FVRRGQPFTIILYF
7/8b A55LSSRRLFVRRGQPF
7/8 E49EHHTKALSSRRLF
7 R46NNEEHHTKALSSRR

Figure 3 Further definition of the cdb3 binding site using overlapping synthetic P4.2 peptides

Analysis of cdb3 binding to these peptides is as described in Figure 1. The location of P4.2 peptides is indicated. Underlined sequence contains residues required for cdb3 binding.

Table 2 Competitive inhibition of P4.2 binding to cdb3 by synthetic P4.2 peptides

Cdb3 was biotinylated and bound to streptavidin-coated 96-well EIA microtitre plates, as described in the Experimental section. This was followed by the simultaneous addition of purified human erythrocyte P4.2 and synthetic P4.2 peptides previously identified as specifically binding to cdb3 (see Figure 1) at molar ratios of 0.2–55 mol of peptide/mol of P4.2. P4.2 binding was detected using anti-P4.2-(479–488) antibody which recognizes human erythrocyte P4.2 but not the synthetic peptides (see Table 1) and AP-conjugated goat anti-rabbit IgG (H + L) with appropriate colour reagents, as described in the Experimental section. Results are presented as absorbance units at 405 nm. P8 and, to a lesser extent, P41 competitively inhibited P4.2 binding to cdb3 (indicated by **bold** rows), as demonstrated by decreasing A_{405} with increasing peptide/P4.2 molar ratio.

Peptide	Peptide/P4.2 molar ratio ...	A_{405}					
		0.2	1.5	3	14	28	55
8		0.84	1.00	0.88	0.64	0.52	0.42
22		0.93	1.43	1.38	1.07	1.20	1.46
27		1.01	1.08	1.02	0.85	0.97	0.85
41		0.99	0.97	0.85	0.83	0.71	0.65
48		1.00	1.12	0.98	1.10	1.23	1.41

Requirement for an RR motif in the cdb3 binding site

To define the cdb3 binding site in P8 further, overlapping peptides were tested using the *in vitro* binding assay. As shown in Figure 3, cdb3 also bound to P7/8b, but much less strongly than to P8. This suggests that a cdb3 binding site is contained within V63RRGQPFTIILYF and that R64R, not R59R, is essential for cdb3 binding. Replacement of R64R with R64G, G64R or G64G almost completely abolished cdb3 binding, further suggesting that R64R is essential for cdb3 binding.

Figure 4 Cdb3 binding to P4.2 tryptic peptides

(a) Purified human erythrocyte P4.2 was subjected to limited trypsin treatment [P4.2/trypsin 50:1 (w/w)] and the proteolytic fragments were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose. Cdb3 binding to the P4.2 proteolytic fragments was as described in the Experimental section. Human red cell P4.2 (HrP4.2) (lane 1) and HrP4.2 tryptic fragments (lane 2) were detected with anti-P4.2 72 kDa antibody (see Table 1). Cdb3 bound to the P4.2 fragments (lane 3) was detected with an anti-cdb3 antibody (see Table 1). The molecular mass of tryptic fragments (indicated on the left) was deduced using molecular-mass standards. (b) Summary of cdb3 binding to P4.2 tryptic fragments shown in (a). The identity of the peptide fragments was verified using anti-P4.2-(96–105) antibody which recognizes an N-terminal epitope approx. 60 residues downstream of the putative cdb3 binding site (see Table 1). Cdb3 binds only to the 23 kDa P4.2 proteolytic fragment which contains the V⁶³RRGQPFTIILYF site. Note that the P4.2 sequence is numbered as described by Korsgren et al. [11] to be consistent with their tryptic fragment map; in this numbering system the putative cdb3 binding site begins at residue 33.

Competitive inhibition of binding of purified human erythrocyte P4.2 to cdb3 by synthetic P4.2 peptides

The five synthetic P4.2 peptides that exhibited specific binding to cdb3 (P8, P22, P27, P41, P48) were tested for their ability to competitively inhibit binding of purified human erythrocyte P4.2 to cdb3. Only P8 and, to a lesser extent, P41 competitively inhibited P4.2 binding to cdb3 (Table 2). P22 and P48 increased P4.2 binding to cdb3 in this assay, the reasons for which are not known but may involve a peptide-induced conformational change in P4.2 that increases cdb3 binding.

Cdb3 binding to P4.2 tryptic peptides

Addition of cdb3 to nitrocellulose blots containing immobilized P4.2 tryptic fragments resulted in binding to an approx. 23 kDa peptide identified by Dotimas et al. [15] as being derived from the P4.2 N-terminus (Figure 4). Only the 23 kDa fragment contains the V⁶³RRGQPFTIILYF cdb3 binding site. We verified the identity of the 23 kDa fragment using anti-P4.2-(96–105) (see

HRP4.2	NNEEHHTKALSSRRLFVRRGQPFTIILYF	75
MRP4.2	NNEEHYTKAISSQHLTLRRGQSFTITLNF	45
GTGLC	NGRDHRTADLCRERLVLRRGQPFWLTLHF	44
HTGLC	NGRDHHTADLCREKLVVRRGQPFWLTLHF	45
MTGLC	NGRDHHTADLCQEKLLVRRGQFRRLTLYF	45
CTGLC	NGREHRTTEEMGSQQLVVRRGQPFTITLNF	53
HF13A	NKVDHHTDKYENNKLIIVRRGQSFYVQIDF	88
HTGLK	NRREHHTDEYEYDELIVRRGQPFHM-LLL	152
RTGLK	NRREHHTDEFYDELILRRGQPFHI-ILF	160
RPTGL	NAAAHHTIEYQTQKLVLRRGQIFSLKVM-	46
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Figure 5 Multiple sequence alignment of human reticulocyte (HR) and mouse reticulocyte (MR) P4.2 [17] and representative transglutaminases

The representative transglutaminases were: GTGLC, guinea-pig liver transglutaminase C; HTGLC, human transglutaminase C; MTGLC, mouse transglutaminase C; CTGLC, chicken transglutaminase C; HF13A, human coagulation factor 13 subunit a; HTGLK, human transglutaminase K; RTGLK, rat transglutaminase K; RPTGL, rat prostate transglutaminase. Alignment was achieved using the CLUSTAL program in PCGENE (Intelligenetics, Mountain View, CA, U.S.A.). An asterisk indicates an identical residue and a bold point a conservative substitution. The RR motif identified as essential for cdb3 binding is in bold type.

Table 1). This antibody recognized the 23, 35 and 17 kDa tryptic fragments, but not the other tryptic fragments, demonstrating that they are derived from the N-terminus, confirming the assignment of Dotimas et al. [15]. In some, but not all, experiments, we also observed faint cdb3 binding to an approx. 25 kDa tryptic peptide (not shown). This peptide is a minor component of the tryptic digest [at a trypsin/P4.2 ratio of 1:50 (w/w)] and is distinct from the 25.5 kDa peptide, which is a major tryptic fragment under these conditions. The location of this 25 kDa peptide in P4.2 is not known. It should be noted that, in our laboratory, limited treatment of P4.2 with trypsin at a trypsin/P4.2 ratio of 1:50 (w/w) did not generate observable amounts of the N-terminal 40 kDa peptide or C-terminal 30 kDa peptide described by Dotimas et al. [15].

Conservation of the cdb3 binding site

As shown in Figure 5, the V⁶³RRGQPFTIILYF cdb3 binding site is highly conserved between human and mouse erythrocyte P4.2, as well as between P4.2 and transglutaminases. At present, it is unclear what role, if any, a potential cdb3 binding site would have in transglutaminase function.

DISCUSSION

We have identified a cdb3 binding site in human erythrocyte P4.2 contained within the domain V⁶³RRGQPFTIILYF, where the motif R⁶⁴R is essential for binding. This analysis was based on binding *in vitro* of synthetic P4.2 peptides to cdb3, the ability of synthetic peptides to inhibit competitively binding of purified human erythrocyte P4.2 to cdb3 *in vitro*, and the binding of biotinylated cdb3 to P4.2 tryptic peptides *in vitro*. Binding of a synthetic P4.2 peptide containing the cdb3 binding site (P8; L⁶¹FVRRGQPFTIILYF) was saturable with a K_d of 0.16 μM and a predicted maximum binding capacity of 0.56 mol of cdb3 monomer/mol of P8. This binding is similar to, and within experimental error of, that previously reported for cdb3 binding to purified human erythrocyte P4.2 [7]. The V⁶³RRGQPFTIILYF cdb3 binding site is located within exon II which is not alternatively spliced and is therefore predicted to be present in all P4.2 isoforms [2,9]. Furthermore, this site is highly conserved between human and mouse P4.2 [18]. The

V⁶³RRGQPFTIILYF cdb3 binding site is located near a pair of potential protein kinase C phosphorylation sites (at residues 57–58) suggesting that P4.2 binding to cdb3 could be affected by phosphorylation state, although this hypothesis has yet to be tested.

The V⁶³RRGQPFTIILYF site contains the basic R⁶⁴R motif suggesting that the P4.2 binding site in cdb3 might be in an acidic region. Supporting this possibility we found that a mutation in the cdb3 acidic domain resulting in the substitution Glu-40 → Lys (band 3^{MONTAFIORE} [19]) is associated with 88 % erythrocyte P4.2 deficiency. However, another band-3 variant, band 3^{TUSCALOOSA} [20], where the mutation occurs closer to the band-3/membrane interface (Pro-327 → Arg), is also associated with a partial (30 %) P4.2 deficiency. This suggests that the Pro-327 → Arg mutation alters band-3 conformation in a way that affects P4.2 binding to V⁶³RRGQPFTIILYF or alternatively that there is more than one P4.2 binding site in band 3.

A mutation has been identified in erythrocytes from anaemic patients of Japanese ancestry that is associated with 99 % P4.2 deficiency (P4.2^{NIPPON}) [2]. The mutation results in the substitution of Ala-142 → Thr. This site, contained within alternatively spliced exon III in human erythrocyte P4.2 [2], is unrelated to the V⁶³RRGQPFTIILYF cdb3 binding site reported here, suggesting that the P4.2 deficiency in P4.2^{NIPPON} is not the result of defective associations with cdb3. Consistent with this speculation, the synthetic P4.2 peptide S¹³¹WTISVT-TPADTVIGHYSL, containing the P4.2^{NIPPON} mutation (Ala-142 → Thr), does not affect P4.2 binding to cdb3 *in vitro* (not shown).

Human erythrocyte cdb3 contains binding sites for other proteins including ankyrin, protein 4.1, haemoglobin, hemichromes, phosphofructokinase, aldolase and glyceraldehyde-3-phosphate dehydrogenase [8]. The cdb3 binding site has been defined in ankyrin and protein 4.1; in ankyrin, it is contained within the 89 kDa N-terminal chymotryptic fragment [21] between Cys-348 and Ser-765 [22]. In protein 4.1, the cdb3 binding site is contained within the 30 kDa chymotryptic N-terminal domain [23]. This domain in protein 4.1 contains a LEEDY motif which binds to a region of opposite charge and similar hydrophobicity in cdb3, L⁽³⁴³⁾RRRY/I⁽³⁸⁶⁾RRRY [24]. This interaction is similar to the binding of P4.2 to cdb3 where both electrostatic and hydrophobic interactions are present in the respective binding sites; for example, in P4.2, the basic motif R⁶⁴R is flanked by hydrophobic residues L⁶¹FP⁶⁸FIIL.

In summary, we have identified a cdb3 binding site in human erythrocyte P4.2 contained within V⁶³RRGQPFTIILYF where R⁶⁴R is essential for cdb3 binding. This identification does not preclude the possibility that other sites in P4.2 may participate in or contribute to cdb3 binding. The V⁶³RRGQPFTIILYF cdb3 binding site is highly conserved in human and mouse erythrocyte P4.2 as well as in transglutaminase proteins from which P4.2 is evolutionarily derived.

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