Identification of a Critical Ankyrin-binding Loop on the Cytoplasmic Domain of Erythrocyte Membrane Band 3 by Crystal Structure Analysis and Site-directed Mutagenesis*

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The cytoplasmic domain of erythrocyte membrane band 3 (cdb3) serves as a center of membrane organization, interacting with such proteins as ankyrin, protein 4.1, protein 4.2, hemoglobin, several glycolytic enzymes, a tyrosine phosphatase, and a tyrosine kinase, p72**. The crystallographic structure of the cdb3 dimer has revealed that residues 175–185 assume a β-hairpin loop similar to a putative ankyrin-binding motif at the cytoplasmic surface of the Na+/K+-ATPase. To test whether this hairpin loop constitutes an ankyrin-binding site on cdb3, we have deleted amino acids 175–185 and substituted the 11-residue loop with a Gly-Gly dipeptide that bridges the deletion without introducing strain into the structure. Although the deletion mutant undergoes the same native conformational changes exhibited by wild type cdb3 and binds other peripheral proteins normally, the mutant exhibits no affinity for ankyrin. This suggests that the exposed β-hairpin turn indeed constitutes a major ankyrin-binding site on cdb3. Other biochemical studies suggest that ankyrin also docks at the NH2-terminus of band 3. Thus, antibodies to the NH2-terminus of cdb3 block ankyrin binding to the cdb3, and ankyrin binding to cdb3 prevents p72** phosphorylation of cdb3 at its NH2-terminus (predominantly at Tyr-8). However, a truncation mutant of cdb3 lacking the NH2-terminal 50 residues displays the same binding affinity as wild type cdb3. These data thus suggest that the NH2-terminus of cdb3 is proximal to but not required for the cdb3-ankyrin interaction.

Ankyrin mediates the attachment of a diverse set of membrane spanning proteins to spectrin-based membrane skeletons. Depending on the cell type, ankyrin may bridge between the β subunit of spectrin and the anion exchanger (1), the Na+/K+-ATPase (2, 3), a voltage-dependent Na+ channel (4), or the Na+/Ca2+ exchanger (5). Cell adhesion molecules such as CD44 (6) and L1CAM family members (7, 8), as well as calcium-release channels such as IP3 receptor (9) and ryanodine receptor (10) are also known to associate with ankyrin. Ankyrin is folded into three independent domains that include an 89-kDa NH2-terminal membrane-binding domain, followed by a 62-kDa spectrin-binding domain and a COOH-terminal regulatory domain. The membrane-binding domain of ankyrin consists of 24 tandem repeats of a 33-amino acid motif known as the ankyrin repeat that is involved in protein recognition (11–14). Because ankyrin interacts with a highly diverse group of membrane proteins, much effort has been devoted to identifying the structural features that mediate these interactions (15–18).

The major linkage between the membrane bilayer and spectrin-based cortical skeleton in erythrocytes is mediated by ankyrin binding to band 3. Because previous studies (19–22) aimed at mapping the docking site(s) of ankyrin on the cytoplasmic domain of erythrocyte membrane band 3 (cdb3) were conducted without the benefit of the crystal structure of cdb3, these investigations of necessity led to inexact conclusions regarding regions or linear sequences of cdb3 implicated in ankyrin binding. Thus, modification of Cys-201 and/or Cys-317 of the cytoplasmic domain of band 3 was found to compromise ankyrin binding (19). Monoclonal antibodies against residues 190–203 (20) or 174–186 (21) were also shown to block the ankyrin interaction. Ankyrin association was further found to protect residues 175–186 of cdb3 from proteolysis (21), and chimera analysis also implicated participation of residues 155–195 (22).

Similar studies have suggested a contribution from the NH2-terminus of cdb3 in the ankyrin interaction (20). Evidence for NH2-terminal involvement has come from the observation that kidney cdb3, which lacks residues 1–65, exhibits no affinity for ankyrin (23, 24). In addition, ankyrin association inhibits phosphorylation of tyrosine residues (predominantly Tyr-8) at the NH2-terminus of band 3 (20). It would appear from these considerations that ankyrin may associate with two disparate regions of cdb3, a region near residues 175–190 and sequences near the NH2-terminus.

With the recent publication of the crystal structure of cdb3 (25), it has become possible to ask more precise questions regarding the docking site of ankyrin on cdb3. In this study, we have noted that a stem-loop structure, which is conformationally similar to a proposed ankyrin-binding site on the Na+/K+-ATPase (26), is located within the general region defined by the earlier mapping studies of Willardson et al. (20) and Davis et al. (21). We have, therefore, decided to evaluate whether this stem-loop structure might constitute a critical conformational motif involved in ankyrin-band3 association. Because of previous evidence for NH2-terminal involvement, we have also explored the nature of the participation of the region in ankyrin binding in greater detail.

MATERIALS AND METHODS

Oligonucleotide-directed Mutagenesis—Oligonucleotide-directed mutagenesis was performed using a QuikChange mutagenesis kit (Strat-
agene) according to the manufacturer’s instructions. The following oligonucleotides were synthesized and used for site-directed mutagenesis.

The pH dependence of intrinsic fluorescence emission of cdb3, which consists of prepacked Superdex 200HR 10/30 (Amersham Biosciences). The pH dependence of intrinsic fluorescence emission of cdb3, which more than doubles between pH levels 6.5 and 9.5, was measured at 340 nm in solutions of 50 mM sodium borate, 50 mM sodium phosphate, 70 mM NaCl adjusted to desired pH levels using an Amino-Bowman luminescence spectrometer at an excitation wavelength of 290 nm and slit widths set at 6 nm.

In Vitro Protein-binding Assay—Purified His-tagged wild type or mutant cdb3 was mixed with increasing concentrations of ankyrin at 4 °C overnight in 7.5 mM phosphate buffer containing 10% sucrose, 90 mM KCl, 10 mM imidazole, 0.4 mM phenylmethylsulfonyl fluoride, and 1 mg/ml bovine serum albumin at pH 7. Pre-equilibrated Ni-NTA beads were incubated with the mixture for 30 min at 4 °C and washed six times with buffer. The bound complexes of cdb3 were eluted with 200 mM imidazole. The quantity of bound ankyrin was then evaluated by either a quantitative dot-blot assay using an anti-ankyrin antibody, or by measuring the radioactive counts in the eluate when—

Peptide Inhibition of Ankyrin Binding—Peptides corresponding to residues 175–185 of human cd3b were synthesized by SynPep (Dublin, CA). An additional cysteine was added to the NH2 and COOH termini of the peptide for subsequent formation of a disulfide bridge between the ends of the peptide in an effort to mimic the hairpin loop seen in the crystal structure. Increasing concentrations of peptide were then mixed with ankyrin prior to addition of cdb3, and the binding assay was performed as described above.

RESULTS

Characterization of a Deletion Mutant of cd3b Lacking a β-hairpin Loop (Residues 175–185)—As noted in the Introduction, the proposed ankyrin-binding site on the CD3 complex of the Na+/K+ ATPase (26) appears structurally similar to a β-hairpin loop revealed in the crystal structure of cd3b (Fig. 1). To evaluate whether this hairpin loop comprising residues 175–185 of cd3b might serve as an ankyrin-binding site, the residues forming the hairpin loop were deleted and a diglycine bridge was substituted in its place. Thus, based on the 4.9 Å distance separating amino acids 175 and 185 in the crystal structure, two glycines were calculated to optimally span the gap without introducing strain into the mutated protein molecule. This deletion mutant was then expressed in E. coli and purified to homogeneity for further characterization.

To confirm that the conformation of the above deletion mutant was not globally perturbed, the reversible pH-dependent conformational change characteristic of native cd3b was examined (30, 31). Thus, as the pH is raised from 6.5 to 9.5, the Stokes radius of native cd3b enlarges by more than 11 Å and the intrinsic fluorescence, which is highly quenched at lower pH, more than doubles. As seen in Fig. 2A, titration of the deletion mutant from pH 6.5 to 9.5 yields the expected fluorescence increase of 2-fold. Further, comparison of the Stokes radius increase of the mutant and wild type cd3b as a function of increasing pH reveals the same dimensional changes in both proteins (Fig. 2B). These data demonstrate that the deletion mutant retains the same structural properties as wild type cd3b.

To further establish that truncation of the β-hairpin loop causes only a localized change in cd3b structure, the interaction of cd3b with protein 4.1, a second major peripheral protein ligand of cd3b was examined. For this purpose, recombinant GST-30 kDa 4.1 was expressed and purified in E. coli. By means of a His tag pull-down assay, which purified His-tagged cd3b was allowed to associate with GST-30 kDa 4.1 and the complex was pelleted with the help of Ni2+ beads, the affinity of mutated cd3b for 4.1 was examined. As shown in Fig. 2C, the amount of bound protein 4.1 associated with mutant cd3b, as measured from the GST activity in the pellet, was the same as the quantity of protein 4.1 associating with wild type cd3b, confirming that deletion of the β-hairpin loop induces no global change in cd3b conformation. Further, glyceraldehydes-3-phosphate dehydrogenase, a glycolytic enzyme that binds to cd3b and becomes inhibited upon binding, was also examined for any change in interaction with the mutant cd3b. As demonstrated in the customary glyceraldehyde-3-phosphate dehydrogenase inhibition assay (33), both wild type and mutant cd3b were able to inhibit the enzyme >90% with the same inhibition constant (data not shown). Taken together, we conclude that any impact of loop deletion on ankyrin binding must be attributed to a direct modification of the attachment site of ankyrin and not to
Evaluation of the Affinity of Ankyrin for the Loop Deletion Mutant of cdb3—Binding of ankyrin to wild type and mutant cdb3 was also evaluated by a His tag pull-down assay similar to that used for analysis of protein 4.1 binding. Thus, His-tagged cdb3 was incubated with a fusion construct of GST linked to the D3/D4 domains of the membrane-binding domain of ankyrin (a construct of ankyrin comprising ankyrin repeats 13–24 that has been frequently used to study ankyrin-band 3 interactions (29, 34–36)) and any complexes formed were pelleted by collection of the His-tagged cdb3 on Ni$_2$H$_{110}$01 beads. The content of pelleted GST-46.5 kDa ankyrin was then quantitated by measuring GST activity in the pellet. As shown in Fig. 3A, the binding curve for the association of the GST-ankyrin fusion construct with His-tagged cdb3 shows saturation with an apparent dissociation constant of 400 nM. Binding was found to be specific because addition of excess unlabeled ankyrin competitively blocked the interaction and because heat denaturation of the 46.5-kDa ankyrin eliminated all association (data not shown). Importantly, binding of the 175–185 deletion mutant to ankyrin was not distinguishable from background, suggesting that the deleted loop is critical for ankyrin association.

Further, when the same analysis was performed by a dot-blot assay using His-tagged cdb3 and unmodified 46.5 kDa ankyrin, a qualitatively similar result was obtained (Fig. 3B). These results, together with the fact that the 175–185 deletion mutant of cdb3 is functionally impaired (37, 38), suggest that this loop is involved in the interaction with ankyrin.

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a general perturbation of cdb3 structure.

FIG. 2. A, comparison of the pH dependence of the intrinsic fluorescence of purified recombinant wild type and mutant (residues 175–185 replaced with a diglycine bridge) human cdb3. Both wild type and mutant cdb3 were dissolved in 50 mM sodium phosphate, 50 mM sodium borate, 70 mM NaCl, pre-adjusted to the desired pH. The relative magnitude of the fluorescence emission at 334 nm (λex, 290 nm) is plotted as a function of pH. B, comparison of the change in Stokes radius of recombinant wild type and mutant cdb3 with pH. Purified wild type and deletion mutant cdb3 (1 mg/ml) were exchanged into 50 mM sodium phosphate, 50 mM sodium borate, 70 mM NaCl, 1 mM EDTA, 0.2 mM dithiothreitol, and 1 mM NaN$_3$ adjusted to the desired pH values. The protein samples (200 μl) were then chromatographed on a prepacked Superdex 200 HR 10/30 column (10 × 300 cm) pre-equilibrated in the same buffer. The Stokes radii were calibrated as described previously (27). C, comparison of GST-30 kDa protein 4.1 binding to wild type and mutant cdb3. His-tagged wild type or mutant cdb3 were incubated with increasing amounts of GST-30 kDa protein 4.1 at 4 °C overnight in isotonic phosphate buffer, pH 7.4. The His-tagged cdb3-GST-30 kDa protein 4.1 complexes were then collected on Ni-NTA agarose beads for 30 min, and the beads were washed 6 times with 6 volumes of buffer and eluted with 250 mM imidazole. GST activity associated with the pelleted protein 4.1 was then measured by following absorbance at 340 nm for 1 h at room temperature upon addition of 1 mM 1-chloro-2,4-dinitrobenzene/1 mM reduced glutathione.
data suggest that the β-hairpin loop on cdb3 plays a critical role in the ankyrin interaction.

Although previous studies have demonstrated that 46.5 kDa ankyrin can bind cdb3 with high affinity (29) and even drive band 3 into tetramers, much like intact ankyrin (34), the question still remained whether the full-length 220 kDa ankyrin, with its twelve additional 33 amino acid repeats, might be less dependent on the β-hairpin loop for binding than 46.5 kDa ankyrin, which lacks the first half of its membrane-binding domain. To address this issue, we purified intact ankyrin from the red cell membrane and radiolabelled it with 125I-Bolton-Hunter reagent. Following incubation with His-tagged cdb3, the resulting ankyrin-cdb3 complexes were pulled-down using Ni2+ beads, and bound ankyrin was quantitated by measuring radioactivity in the pellet.

In contrast to our expectations from previous studies (20, 23, 24), cdb3 lacking residues 1–50 exhibited the same binding affinity for 46.5 kDa ankyrin as wild type cdb3 (Fig. 3), suggesting that the NH2 terminus may not be essential for ankyrin association. Nevertheless, because the previous studies were conducted with intact ankyrin rather than truncated 46.5 kDa ankyrin, and because the earlier data still seemed compelling, it was decided to re-evaluate the affinity of the 1–50 deletion mutant of cdb3, but this time for the full-length ankyrin (i.e. the 220-kDa ankyrin might still require the NH2-terminal 50 residues of cdb3 as unstructured and can be eliminated without perturbing any other regions of the polypeptide (25). Therefore, residues 1–50 of cdb3 were deleted, and the resulting truncated cdb3 was evaluated for ankyrin affinity.

To further support the hypothesis that residues 175–185 participate directly and prominently in ankyrin binding, the 11 amino acid peptide corresponding to residues 175–185 was synthesized, only in this case a cysteine residue was added to each end of the peptide to allow for disulfide-mediated cyclization of the peptide. Cyclization was considered important as a means of trying to mimic the 3-dimensional structure of the β-hairpin loop in situ. The resulting looped peptide was then tested for its ability to inhibit binding of 46.5 kDa ankyrin to cdb3. As seen in Fig. 5, the synthetic peptide was indeed able to inhibit ankyrin-cdb3 association. Nevertheless, because the previous studies were suggesting that the NH2 terminus may not be essential for ankyrin association, we conducted experiments using 46.5 kDa ankyrin instead of intact ankyrin and found that all three lines of evidence for NH2-terminal involvement could be replicated (i.e. kidney band 3 does not bind 46.5 kDa ankyrin). 46.5 kDa ankyrin inhibits tyrosine phosphorylation at the NH2 terminus of cdb3, and antibodies to the NH2 terminus of cdb3 block 46.5 kDa ankyrin binding (data not shown)). These results argue strongly that although residues 1–50 of cdb3 may not physically participate in ankyrin binding, they must reside sufficiently proximal to the ankyrin-binding site on cdb3 that antibody binding to the NH2 terminus prevents ankyrin association, and ankyrin association at the β-hairpin loop sterically obstructs phosphorylation of cdb3 on Tyr-8. Examination of the crystal structure of cdb3 demonstrates that this steric overlap is entirely reasonable.

**DISCUSSION**

We have presented evidence that the β-hairpin loop comprising residues 175–185 of cdb3 constitutes a major ankyrin-binding site on the erythrocyte membrane. Although previous studies (19–22) have suggested the involvement of a mid-re-
Deletion mutagenesis of the NH$_2$ terminus of cdb3 further demonstrated that the first 50 residues of the polypeptide are not directly involved in ankyrin binding. However, the observations that blocking of this sequence by peptide-specific Fabs totally prevents ankyrin binding, and ankyrin binding to the \( \beta \)-hairpin loop inhibits phosphorylation of Tyr-8 argues strongly that the NH$_2$ terminus and the ankyrin-binding site lie near each other. Curiously, prediction of this steric interference would not have been obvious from the crystal structure because the first 55 residues of cdb3 are too flexible to be resolved and because the \( \beta \)-hairpin loop lies ~50 Å from residue 55 (i.e. the site where the flexible NH$_2$-terminal sequence leaves the body of cdb3). Nevertheless, a close physical proximity must be real because ankyrin also competes with other peripheral proteins that bind at the NH$_2$ terminus of band 3 (38). Because the NH$_2$ terminus of cdb3 is not only required for stable interaction with protein 4.1 (38) but also for association with various glycolytic enzymes (33, 39) and p72$^{\alpha b}$ (40, 41), it is also conceivable that binding of ankyrin to cdb3 might be involved in regulation of other protein interactions at the NH$_2$ terminus.

The absence of a requirement for residues 1–50 in ankyrin binding raises the question of why kidney cdb3, which lacks residues 1–50, fails to bind ankyrin. Analysis of the crystal structure, however, demonstrates that residues 57–65 constitute a central strand in an eight-stranded \( \beta \) sheet that extends through the middle of cdb3. Although deletion of residues 1–50 can be argued to have little or no impact on protein conformation, removal of residues 1–65 should force rearrangement of the packing of the entire domain. Thus, based on crystallographic considerations, kidney cdb3 should have a somewhat different conformation from erythrocyte cdb3. Consistent with this speculation, it has been reported that kanadapin interacts with kidney, but not erythrocyte band 3 (42), whereas the glycolytic enzymes, ankyrin, and protein 4.1 associate with erythrocyte but not kidney band 3 (24).

It is interesting to note that the binding properties of intact ankyrin to band 3 are reasonably well replicated by the binding properties of 46.5 kDa ankyrin to cdb3. Not only do both polypeptides require association at the \( \beta \)-hairpin loop, but preparation appears to involve docking with the NH$_2$ terminus, even though both are inhibited by Fab5 directed at this sequence, and both prevent phosphorylation of Tyr-8. Previous studies (35) have suggested that ankyrin contains one cdb3-binding site on its second set of 6 ankyrin repeats (subdomain 2) and another binding site somewhere within repeats 13–24 (subdomains 3 and 4, i.e. the 46.5-kDa ankyrin. Based on the above similarities in binding properties, it is tempting to speculate that most of the interfacial contacts may occur between subdomains 3 and 4 and cdb3 and that docking with subdomain 2 may involve a more limited surface area.

Comparison of the proposed Na$^+$/K$^+$-ATPase and cdb3-binding sites for ankyrin might suggest that a \( \beta \)-hairpin loop constitutes a common ankyrin-binding motif on the cytoplasmic domains of multiple membrane-spanning proteins. Even though the loop on cdb3 shares no sequence homology with the loop on the Na$^+$/K$^+$-ATPase, cdb3 still inhibits the binding of ankyrin to the Na$^+$/K$^+$-ATPase (43). We interpret this to suggest that a conformational fit rather than sequence complementarity at the binding interface contributes primarily to complex formation. Indeed, our inability to compromise ankyrin-binding affinity by mutating both charged residues in the \( \beta \)-hairpin loop (data not shown) is consistent with the more dominant role played by conformational fit in stabilizing this interaction. As other ankyrin-binding structures become available, it will be interesting to examine whether similar exposed loops constitute the sites of association.

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