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Expression, purification, and characterization of the functional dimeric cytoplasmic domain of human erythrocyte band 3 in *Escherichia coli*

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Abstract

The cytoplasmic domain of the human erythrocyte membrane protein, band 3 (cdb3), contains binding sites for hemoglobin, several glycolytic enzymes, band 4.1, band 4.2, and ankyrin, and constitutes the major linkage between the membrane skeleton and the membrane. Although erythrocyte cdb3 has been partially purified from proteolyzed red blood cells, further separation of the water-soluble 43-kDa and 41-kDa proteolytic fragments has never been achieved. In order to obtain pure cdb3 for crystallization and site-directed mutagenesis studies, we constructed an expression plasmid that has a tandemly linked T7 promoter placed upstream of the N-terminal 379 amino acids of the erythrocyte band 3 gene. Comparison of several *Escherichia coli* strains led to the selection of the BL21 (DE3) strain containing the pLysS plasmid as the best host for efficient production of cdb3. About 10 mg of recombinant cdb3 can be easily purified from 4 L of *E. coli* culture in two simple steps. Comparison of cdb3 released from the red blood cell by proteolysis with recombinant cdb3 reveals that both have the same N-terminal sequence, secondary structure, and pH-dependent conformational change. The purified recombinant cdb3 is also a soluble stable dimer with the same Stokes radius as erythrocyte cdb3. The affinities of the two forms of cdb3 for ankyrin are essentially identical; however, recombinant cdb3 with its unblocked N-terminus exhibits a slightly lower affinity for aldolase.

Keywords: band 3 conformation; cytoplasmic domain of band 3; inclusion body; protein expression in *Escherichia coli*; T7 promoter

Band 3 is the predominant polypeptide of human erythrocyte membrane, constituting ~25% of the total membrane protein. Band 3 has a monomer molecular weight of ~100 kDa and is composed of two domains easily separated by proteolytic digestion. The externally exposed 57-kDa transmembrane domain functions as an anion exchanger, while the 43-kDa dimeric cytoplasmic domain (cdb3) serves to link numerous cytoplasmic and cytoskeletal proteins to the membrane (Low, 1986). Those polypeptides thought to associate with cdb3 in vivo include ankyrin (Bennett & Stenbuck, 1980; Low et al., 1991); band 4.2 (Korsgren & Cohen, 1988); band 4.1 (Pasternak et al., 1985; Danilov et al., 1990); the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase (Tsai et al., 1982), aldolase (Murthy et al., 1981), and phosphofructokinase (Jenkins et al., 1984); hemoglobin (Walder et al., 1984); and hemichromes (Waugh & Low, 1985). The two major domains of band 3 are connected by a flexible, proteolytically sensitive peptide of at least 20 amino acids that can be cleaved at several sites to yield cdb3 fragments of various lengths.

The several functions of cdb3 all relate to its interaction with peripheral proteins. Cdb3 participates in the control of membrane mechanical stability via its pH-dependent association with ankyrin (Thevenin & Low, 1990; Low et al., 1991). Perturbations of this interaction, whether by artificial means or through natural mutation, can dramatically affect the rigidity and stability of the cell (Liu et al., 1990; Low et al., 1991). Cdb3 also binds and regulates the activities of several glycolytic enzymes by associating reversibly with the enzymes in an inhibitory

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complex (Low et al., 1987). Modulation of this interaction by phosphorylation of Tyr 8 and 21 on cdb3 can significantly affect glycolytic rates in vivo (Harrison et al., 1991). The clustering of band 3 in the membrane via hemichrome binding to the N-terminus of cdb3 mediates the removal of aged and damaged red blood cells from circulation (Low et al., 1985), and association of native hemoglobin with band 3 may regulate certain structural properties of both polypeptides (Low & Kannan, 1989). Although the functions of the band 4.1 and 4.2 interactions have not yet been established, they are also likely to be important, since most of the cellular band 4.1 (Pasternak et al., 1985; Danilov et al., 1990) and band 4.2 (Korsgren & Cohen, 1988) appears to reside on band 3.

Compared to functional data on cdb3, structural information on the protein is still relatively lacking. Although erythrocyte cdb3 has been partially purified, the component proteolytic fragments (43, 41 kDa) have never been separated under nondenaturing conditions (Low, 1986). Although a major pH-dependent conformational equilibrium in cdb3 can be detected by virtually any biophysical technique (Appell & Low, 1981; Low et al., 1984), the nature of the structural change and the residues involved are largely unknown. And even though the binding sites of most peripheral protein ligands have been mapped to specific regions of cdb3 (Murthy et al., 1981, 1984; Davis et al., 1989; Willardson et al., 1989; Lombardo et al., 1992), the relative positions of these regions in the native folded structure are still an enigma. Therefore, in order to begin to elucidate the structural properties of cdb3 by X-ray crystallography and site-directed mutagenesis, we have undertaken to express large quantities of the N-terminal 379 amino acids of band 3 in *Escherichia coli*. Here we report the construction of a stable expression vector of cdb3 in *E. coli* and describe its use in generating milligram quantities of the purified protein. We further describe a simple two-step protocol for isolation of recombinant cdb3 and then demonstrate by several methods that the genetically engineered protein is virtually indistinguishable from native cdb3.

Results and discussion

Expression vector construction

The pT7-7 expression vector contains a T7 polymerase promoter and ribosome binding site in front of an NdeI cloning site. When transformed into *E. coli* strain BL21 (DE3) containing the T7 polymerase gene under control of the lac promoter, expression of the desired cloned gene can be stimulated by the inducer isopropyl β-D-thiogalactopyranoside (IPTG). Figure 1 shows the constructed expression vector, cdb3/T7-7. The polymerase chain reaction (PCR)-amplified cdb3 gene was inserted into NdeI and HindIII sites of the T7-7 vector using the aforementioned restriction enzymes and T4 ligase. The ampicillin-resistant clones were analyzed by digesting with restriction enzymes in order to verify the integrity of the cdb3 gene.

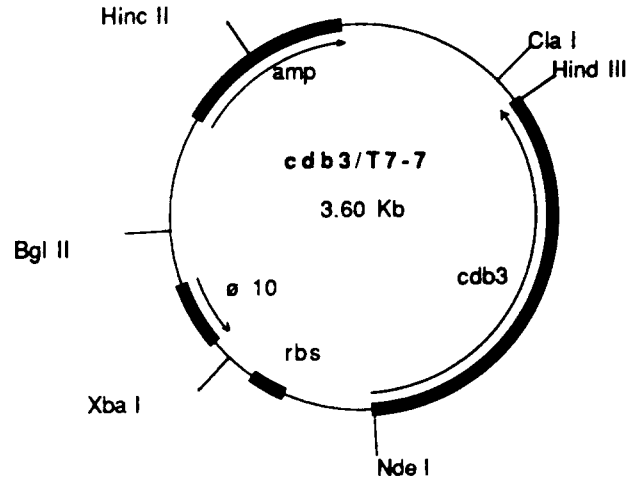

Fig. 1. Construction of the cdb3/T7-7 expression vector. The cdb3 gene encoding residues 1–379 of band 3 was amplified with primers that introduced an NdeI site at its 5′ end and a stop codon followed by a HindIII site at its 3′ end. After insertion into the complementary sites of the pT7-7 vector, the plasmid was introduced into *Escherichia coli* BL21 (DE3) and proliferated in ampicillin-containing medium.

Optimization of the expression conditions

The classic T7-7 expression system (Studier & Moffatt, 1986) uses *E. coli* BL21 (DE3) as the host and 0.4–1 mM IPTG as the inducer. However, even when transformed *E. coli* (DE3) were grown in the absence of inducer, the cdb3/T7-7-transformed cells were found to be unstable, rapidly yielding a culture lacking the plasmid. Closer analysis revealed that low amounts of cdb3 had been expressed in the uninduced cultures and that the synthesized cdb3 was partially degraded. Assuming the weakly expressed cdb3 or T7 polymerase might be at least partly responsible for rejection of the cdb3/T7-7 plasmid, an inhibitor was sought that would inactivate any uninduced T7 polymerase prior to IPTG addition. For this purpose, two different *E. coli* strains containing plasmids expressing either low (pLysS) or high (pLysE) levels of the T7 polymerase inhibitor, T7 lysozyme (Studier et al., 1990; Studier, 1991), were examined for their abilities to retain the cdb3/T7-7 plasmid and express cdb3 upon induction. Both strains were found to serve as stable hosts for the cdb3/T7-7 plasmid; however, they differed in their abilities to produce cdb3 upon induction (Fig. 2). Whereas the strain containing pLysE yielded low amounts of cdb3 following IPTG addition, the strain transformed with pLysS produced substantial quantities of the erythrocyte protein. It is conceivable that the higher levels of T7 lysozyme expression in BL21 (DE3) (pLysE) not only blocked uninduced T7 polymerase activity but also inhib-

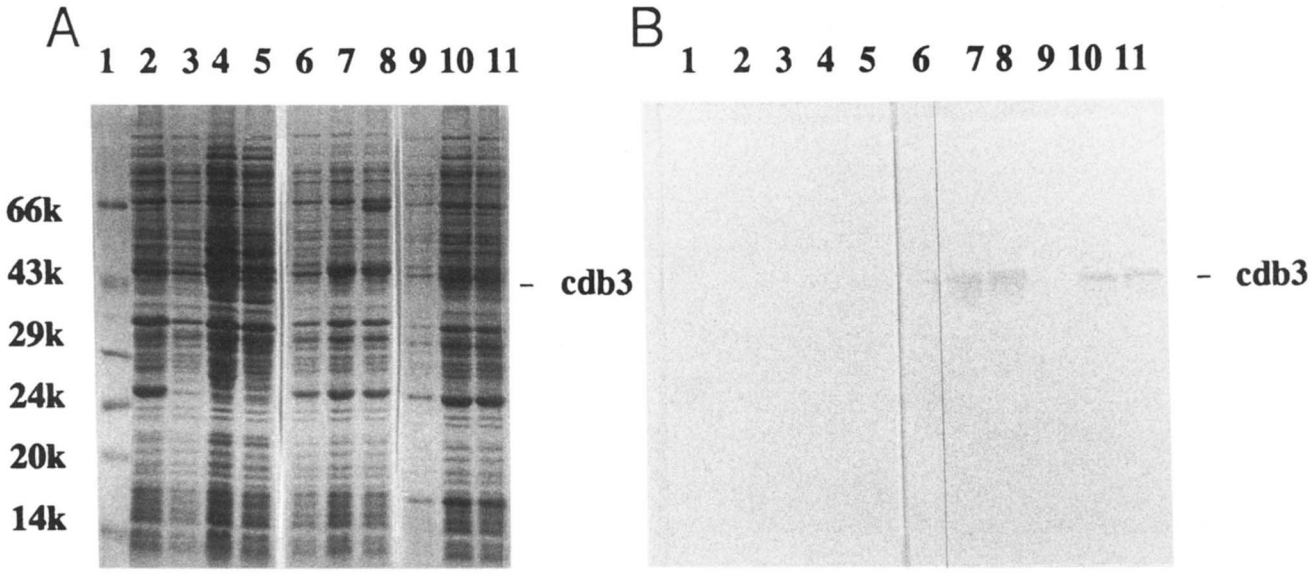

Fig. 2. Selection of *Escherichia coli* host strain for expression of cdb3 using cdb3/T7-7 plasmid. Lane 1 contains molecular weight markers. Lane 2 is BL21 (DE3) strains without cdb3/T7-7 vector. Cdb3/T7-7 plasmid was introduced in BL21 (DE3) strains of *E. coli* containing either no expression vector for T7 lysozyme (lanes 3–5), the pLysS plasmid promoting low level expression of T7 lysozyme (lanes 6–8), or the pLysE plasmid promoting high level expression of T7 lysozyme (lanes 9–11). Lanes 3, 6, and 9 contain the unclarified lysate from uninduced cultures, whereas lanes 4, 5, 7, 8, 10, and 11 contain the corresponding lysates from IPTG-induced cultures. **A:** Coomassie blue-stained gel. **B:** Immunoblot of gel using polyclonal antibodies to human erythrocyte cdb3.

ited a significant fraction of the induced polymerase, thereby reducing cdb3 expression. Others have reported similar results with T7 lysozyme expression (Lin et al., 1990; O'Mahony et al., 1990; Studier, 1990).

Besides vector stability, cdb3 expression was also found to be affected by growth temperature and proteolysis (Babbitt et al., 1990; Lin et al., 1990; Steczko et al., 1991). Although the transfected *E. coli* proliferated faster at 37 °C, most of the cdb3 induced at this temperature aggregated into inclusion bodies. Furthermore, unwanted proteolysis of cdb3 from its parent molecular weight of ~43 kDa to a fragmentation product at ~38 kDa was prominent at higher temperatures (Fig. 3, lanes 8–10 at 31 °C, 15–17 at 37 °C). Inclusion of protease inhibitors phenylmethylsulfonylfluoride (PMSF) (lane 13) or benzamidine (lane 14) in the culture medium caused a little improvement, and although mild inhibition of protein synthesis with 200 μg/mL rifampicin may have eliminated some proteolysis, it also reduced cdb3 expression (lane 12). Because others have reported improved gene expression at lower temperatures with slower induction rates (Lin et al., 1990; Steczko et al., 1991), we elected to examine this variable also. As shown in lanes 1–11 (Fig. 3), as growth temperatures and induction time were lowered, significantly reduced proteolysis was observed with only a minor sacrifice in total yield. For this reason, all further expression studies were conducted for 6 h at a growth temperature of 25 °C.

Purification of cdb3 from E. coli

Because of the co-expression of T7 lysozyme in the cytoplasm of the BL21 (DE3) (LysS) strain, it was possible to lyse the *E. coli* by a mild freeze–thaw procedure using several consecutive immersions into liquid N_2 followed by brief sonication (see Methods). Triton X-100 and 2-mercaptoethanol were found to be essential components of the lysis buffer, since without them the resulting cdb3 was either denatured or disulfide cross-linked to other *E. coli* proteins. Furthermore, the final cdb3 yield in the absence of 2-mercaptoethanol was only $\frac{1}{3}$ the yield in its presence, and many contaminating proteins remained with band 3 through the final purification step. Probably the most useful stratagem in the purification protocol was the addition of DEAE-cellulose directly to the unclarified crude cell lysate. Hoess et al. (1988) have reported that direct addition of the Q-Sepharose (a strong anion exchanger) to *E. coli* lysate can promote release of expressed proteins from inclusion bodies, and we found that recovery of expressed cdb3 improved from ~10% to essentially 100% upon use of DEAE-Sepharose (a weak anion exchanger). Following elution of cdb3 from the DEAE column with a salt gradient and subsequent chromatography in Sephacryl S-300 gel filtration media, a homogeneous preparation of undigested cdb3 can be isolated (Fig. 4). From 4 L of culture medium, 10 mg of purified cdb3 is commonly obtained.

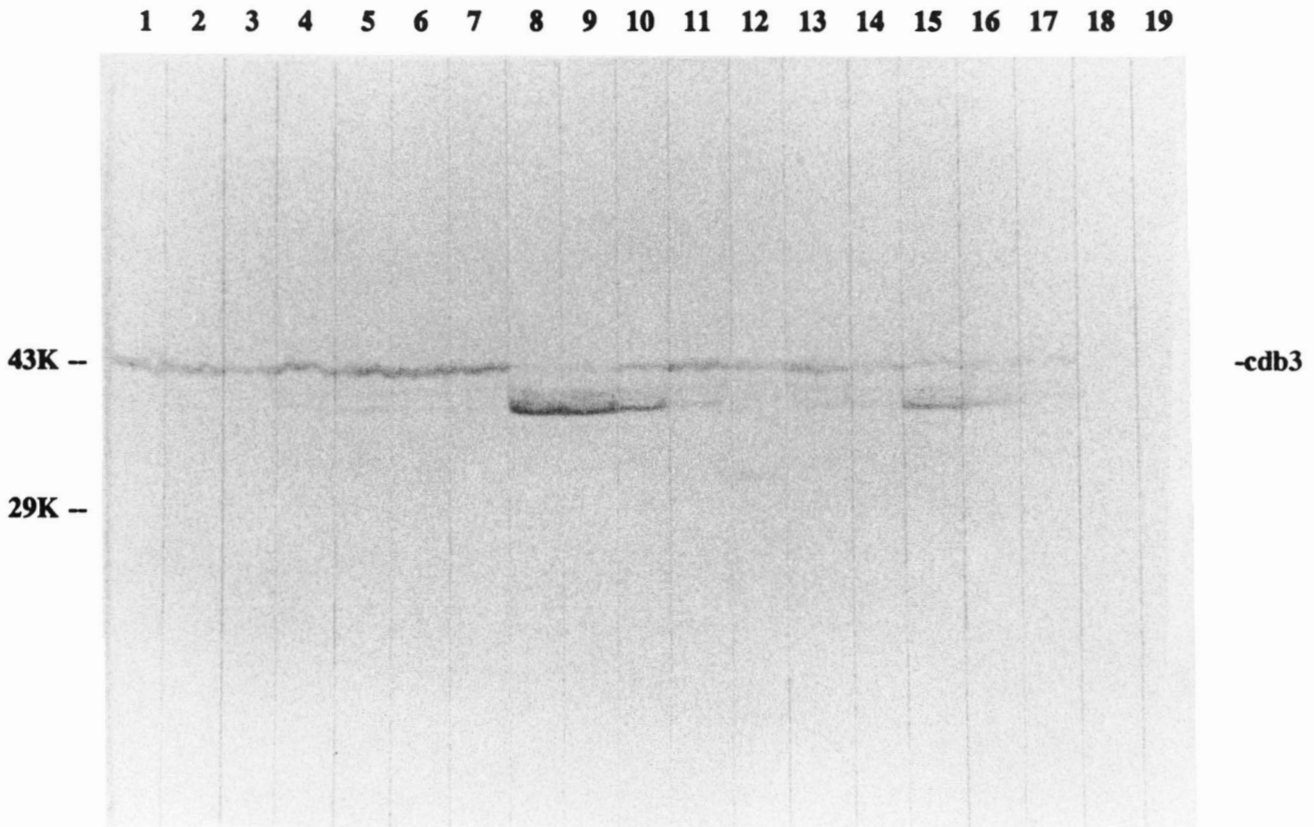

Fig. 3. Optimization of expression conditions for cdb3/T7-7 in *Escherichia coli*. *Escherichia coli* strain BL21 (DE3) (pLysS) transformed with vector cdb3/T7-7 was grown in LB medium under different conditions. After induction with IPTG and lysis, the unclarified lysates were separated electrophoretically in 12% sodium dodecyl sulfate–polyacrylamide gels and immunoblotted with a polyclonal antibody to human erythrocyte cdb3. Cells from the same flask were aliquoted into different culture tubes, induced with 1 mM IPTG, and cultured for the times and at the temperatures indicated below. Lanes 1–3 were grown at 21 °C for 8, 6, and 3 h; lanes 4–7 were grown at 25 °C for 9, 8, 6, and 3 h; lanes 8–11 were grown at 31 °C for 9, 8, 6, and 3 h. The cultures in lanes 12–19 were all grown at 37 °C. Lanes 15, 16, and 17 were proliferated for 4, 3, and 2 h, respectively, whereas lanes 12–14, 18, and 19 were grown for 3 h. Lane 12 contained 200 μg/mL rifampicin, lane 13 contained 200 μg/mL phenylmethylsulfonylfluoride, and lane 14 contained 10 mM benzamidine in LB medium. Negative controls are shown in lane 18 (uninduced) and lane 19 (T7-7 vector lacking cdb3 gene).

Structural characterization of recombinant cdb3

Direct sequencing of the cloned cdb3 by Edman degradation yielded the same N-terminal 18 amino acids found in erythroid band 3. Because there was no increase in the molar recovery of phenyl thiohydantoin amino acids following mild acid hydrolysis to remove N-terminal blocking groups, we conclude that the customary *N*-acetyl modification found quantitatively in erythroid band 3 was not performed by the *E. coli*. The isolated recombinant cdb3 migrated in 12.5% sodium dodecyl sulfate–polyacrylamide gels at its expected molecular weight of 42.5 kDa (Fig. 4), and following electroblotting onto nitrocellulose it stained with anti-human cdb3 antibodies (Willardson et al., 1989). Examination of the circular dichroism spectrum of cloned cdb3 (data not shown) indicated that it was identical to the previously published spectrum of erythroid cdb3 (Appell & Low, 1981; Low et al., 1984), and evaluation of the Stokes radius of the recombinant protein showed it to be a dimer with the same characteristics as its erythroid counterpart (Fig. 5; Low et al., 1984). Most diagnostic of the native state of the cloned protein, however, was the ability of the isolated cdb3 to undergo the unusual conformational change characteristic of red cell cdb3. Not only did recombinant cdb3 reversibly expand from a Stokes radius of ~55 Å to 68 Å between pH 6.5 and 10 (Fig. 5), but the protein's intrinsic fluorescence more than doubled over this same pH range (Fig. 6). The former pH transition is characterized by an apparent pK_a of ~8.8 (Fig. 5), whereas the latter is described by two pK_a's of 7.3 and 9.2 (Fig. 6). Denatured cdb3 was completely unable to undergo these reversible pH transitions. All of these properties are essentially identical in erythrocyte cdb3 (Appell & Low, 1981; Low et al., 1984), suggesting that the recombinant protein is native. Except for the absence of *N*-acetylation, we were

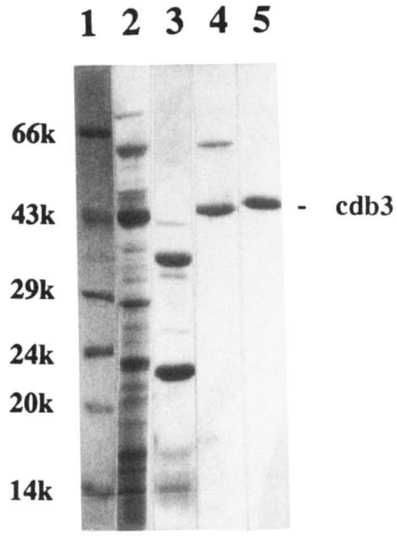

Fig. 4. Coomassie blue-stained gel containing cdb3 at various stages of purification from *Escherichia coli* extract. Samples from different stages in the preparation of cloned cdb3 were subjected to 12% polyacrylamide gel electrophoresis gel according to the method of Laemmli (1970). Lane 1 contains protein molecular weight markers from Sigma; lane 2 contains the crude *E. coli* cell lysate; lane 3 displays the material eluted in the 0.2 M NaCl wash from the DEAE-Sepharose CL-6B column; lane 4 contains the cdb3 fraction eluted between 0.35 and 0.4 M NaCl from the DEAE-Sepharose CL-6B column; and lane 5 is the first peak off the Sephacryl S-300 column.

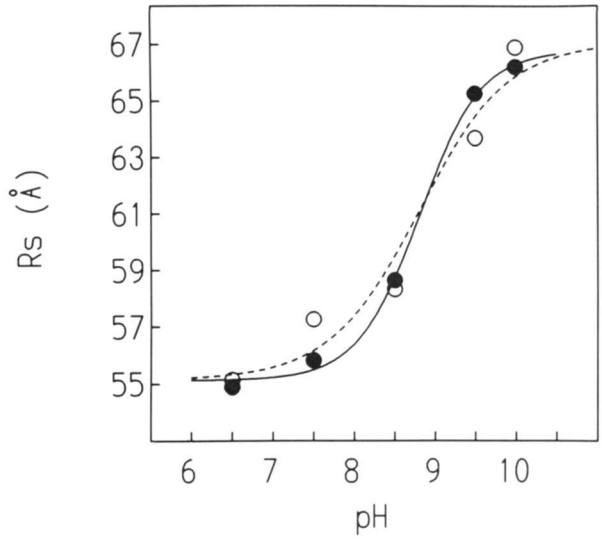

Fig. 5. The Stokes radius (Rs) of cloned cdb3 plotted as a function of pH. Purified recombinant cdb3 (1 mg/mL) was dialyzed overnight against 50 mM sodium phosphate, 50 mM sodium borate, 70 mM NaCl, 1 mM EDTA, 0.2 mM dithiothreitol, and 1 mM NaN_3 at various pHs. The protein (2 mL) was then chromatographed on a Sephacryl S-300 column (2.7 × 117 cm) equilibrated in the same dialysis buffer. The Stokes radius of cdb3 was calibrated using the following standards chromatographed on the same column: horse spleen apoferritin (Rs = 79 Å), bovine liver catalase (Rs = 52 Å), rabbit muscle aldolase (Rs = 46 Å), bovine serum albumin (Rs = 35 Å), and ovalbumin (Rs = 27 Å). The solid curve (●) is the cloned cdb3 and the dashed curve (○) for erythrocyte cdb3 was taken from Low et al. (1984).

unable to detect any structural difference between the cloned and natural protein.

Functional characterization of recombinant cdb3

Aldolase and several other glycolytic enzymes bind to the extreme N-terminus of cdb3 both in vitro (Murthy et al., 1984; Low et al., 1987) and in vivo (Harrison et al., 1991). Importantly, upon association with band 3, these enzymes are reversibly inhibited, allowing for the participation of band 3 in the regulation of glycolysis (Low et al., 1987; Harrison et al., 1991). In studies using a synthetic peptide corresponding to the N-terminus of band 3, Murthy et al. (1981) found that omission of *N*-acetylation reduced the affinity of the peptide for aldolase. Consistent with this model study, we found that nonacetylated recombinant cdb3 bound and inhibited aldolase less avidly than its acetylated erythroid counterpart (Fig. 7). Because the association between the strongly anionic N-terminus of cdb3 and a cationic site on the enzyme is dependent primarily on charge interactions, the 20% decrease in inhibitory potency upon introduction of a positive charge at the N-terminus is perhaps not very surprising. Nevertheless, it is interesting to speculate that the recombinant cdb3 is still a sufficiently potent inhibitor to account for the instability of *E. coli* strains that constitutively express low amounts of cdb3 (see above).

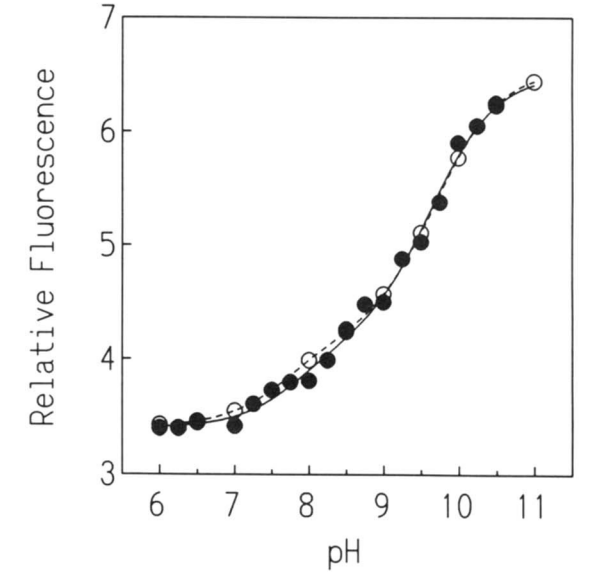

Fig. 6. The pH dependence of the intrinsic fluorescence of purified recombinant cdb3 dissolved in 50 mM sodium phosphate, 50 mM sodium borate, 70 mM NaCl, preadjusted to the desired pH. The final protein concentration in all cases was 37 μg/mL. The relative magnitude of the fluorescence emission at 335 nm (λ_{ex}, 290 nm) is plotted as a function of pH. The experimentally obtained points (●) were fit with a nonlinear least-squares program for a double titration curve. The dashed line displays the corresponding pH profile of cdb3 isolated from human erythrocytes, as described previously (Low et al., 1984).

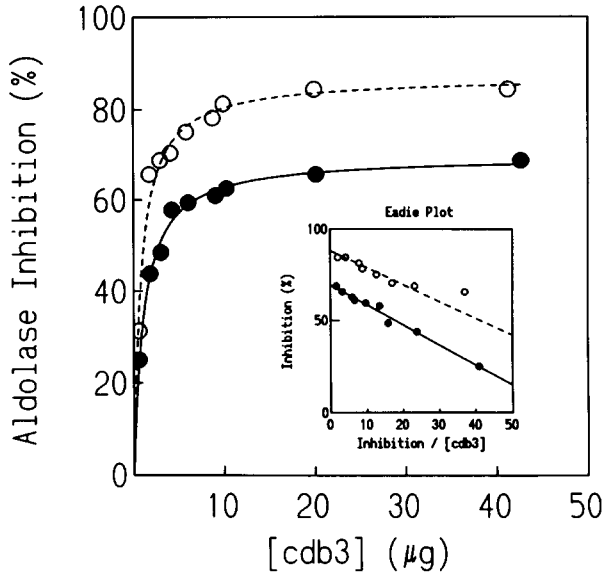

Fig. 7. Comparison of the abilities of recombinant and erythrocyte cdb3 to inhibit aldolase. Increasing amounts of cloned or natural cdb3 were mixed with 2.2 μg rabbit muscle aldolase (Sigma) in a total volume of 1 mL containing 3.5 mM hydrazine sulfate, pH 6.0, and 0.1 mM EDTA. After 5 min incubation, the aldolase–cdb3 solution was added to a cuvette containing 0.1 mL of 12 mM fructose-1,6-bisphosphate plus 2 mL of the above hydrazine–EDTA buffer. The absorbance at 240 nm was then monitored continuously for 5 min, and aldolase activity was calculated. The data in the inset are plotted according to the Eadie–Hofstee equation. Inhibition percentages of aldolase are for the erythrocyte (○, dashed curve) and recombinant cdb3 (●, solid curve), respectively.

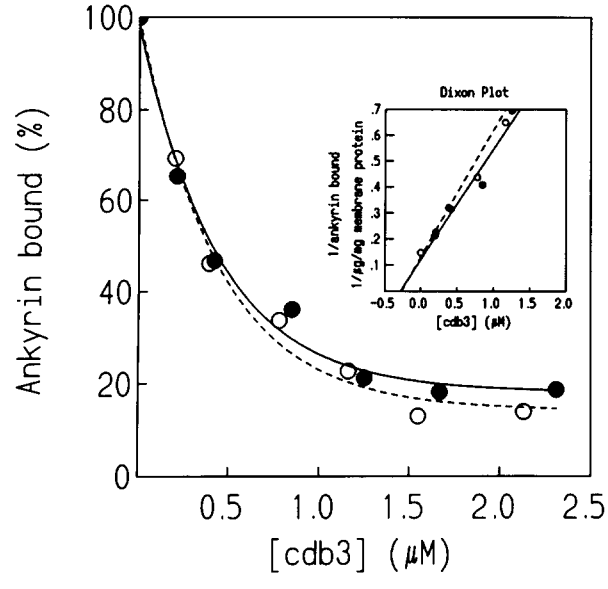

Fig. 8. Comparison of the abilities of recombinant and erythrocyte cdb3 to competitively inhibit ankyrin binding to stripped inside-out erythrocyte membrane vesicles. Inside-out erythrocyte membrane vesicles stripped by 1 M KI (43 μg/mL membrane protein) were incubated 30 min on ice in the presence of various concentrations of cloned (●, solid curve) or erythrocyte (○, dashed curve) cdb3. ^{125}I-labeled ankyrin (4 μg/mL, final concentration) was then added and the mixture was allowed to further incubate 3 h on ice. The mixture was then layered on top of a 600-μL sucrose cushion (155 mM NaCl, 0.2 mM dithiothreitol, 1.2 mg/mL bovine serum albumin, 15% sucrose) and centrifuged at 45,000 × *g* for 40 min. The microfuge tubes were frozen in liquid N_2, and the tips containing the vesicle pellet were cut and counted for ^{125}I-ankyrin. Further details are provided elsewhere (Thevenin & Low, 1990). The results are plotted both as a simple inhibition curve and a Dixon plot (inset). The apparent k_I for both cdb3 preparations is 330 nM.

Probably the most important function of cdb3 is its tethering of the membrane skeleton to the bilayer via an association with ankyrin (Thevenin & Low, 1990; Low et al., 1991). To evaluate whether recombinant cdb3 still retained a native capacity to bind ankyrin, both direct and competitive binding studies were conducted. When a mixture of ankyrin and cloned cdb3 were sedimented at high speed in a sucrose density gradient, the two proteins migrated as a complex, as described previously for erythrocyte cdb3 (data not shown). Furthermore, when the abilities of recombinant and natural cdb3 to competitively inhibit ankyrin binding to inside-out erythrocyte membrane vesicles were compared, no differences could be discerned (Fig. 8). In fact, the resulting inhibition constant (k_I ~330 nM) was nearly identical to that reported previously by Bennett and Stenbuck (1980). Thus, the ankyrin binding properties of genetically engineered cdb3 appear to be totally normal.

In summary, a functional dimeric cytoplasmic domain of erythrocyte band 3 can be expressed in large quantities in a stable strain of *E. coli*. If appropriate cell growth and cdb3 isolation conditions are selected, nearly quantitative recovery of the undigested native protein can be achieved. Except for a small decrease in affinity for glycolytic enzymes due to the absence of *N*-acetylation, no other structural or functional differences from erythrocyte cdb3 could be detected. Therefore, recombinant cdb3 should be useful for crystallization and site-directed mutagenesis studies.

Materials and methods

Materials

Reagents for the PCR and the corresponding thermal cycler were from Perkin-Elmer Cetus Corp. DEAE-Sepharose CL-6B and Sephacryl S-300 were from Pharmacia LKB Biotechnology. Glycolytic enzymes, protein molecular weight markers, and other general chemicals were obtained from Sigma. Growth media for *E. coli* were from Difco Laboratory. IPTG and antibiotics (rifampicin, chloramphenicol, and ampicillin) came from US Biochemical Corp. DNA markers were from BRL, and nitrocellulose was purchased from Schleicher & Schuell. Erythrocyte cdb3 polyclonal antibodies were prepared in our laboratory and preabsorbed with the supernatant of an *E. coli*

(DE3) lysate to neutralize any nonspecific interactions. Electrophoresis reagents and horseradish peroxidase-conjugated anti-rabbit antibodies were from Bio-Rad, and restriction enzymes and T4 ligase were from New England Bio-lab. *Escherichia coli* B21 (DE3) and the derived pLysS- and pLysE-containing strains, as well as the T7-7 vector were gifts of Dr. F.W. Studier (Stony Brook).

Methods

Cdb3 gene amplification

The pHB3-45 clone (Lux et al., 1989) containing the cdb3 complementary DNA (cDNA) was initially cloned into a Bluescript-KS (+) plasmid vector. The cdb3 cDNA (containing the N-terminal 379 amino acids of band 3) was amplified using two specifically designed primers and PCR methodology. The PCR primers were synthesized to yield an NdeI site at the 5′ end and a HindIII site with a TCA stop codon at the 3′ end of the amplified gene. The NdeI/HindIII fragment was then subcloned into the T7 expression vector (pT7-7). The primers used were 5′-TCTCATATGGAGGAGCTGCAGGATG-3′ for the 5′ end, 5′-AGAAAGCTTTCAGAAGAGCTGGC CTGTCTGCTG-3′ for the 3′ end. The reaction solution in the thermal cycler contained 1 μL pHB3-45 (2 μg/mL), 5 μL of both primers (10 μM stock), 16 μL deoxynucleoside triphosphates (1.25 mM stock), 0.5 μL taq DNA polymerase (5 units/μL), 62.5 μL H_2O, and 10 μL 10× PCR kit buffer (Perkin Elmer-Cetus). Negative controls were set up similarly except template DNA was omitted. The PCR parameters used were 1 min at 94 °C, 2 min at 41 °C, and 2 min at 72 °C, continued for 35 cycles. The amplified 1.15-kb cdb3 cDNA was subjected to agarose gel electrophoresis and visualized with ethidium bromide. The 1.15-kb fluorescent band was excised and purified by standard protocols (Sambrook et al., 1989).

Expression vector construction and E. coli (DE3) transformation

Both purified cdb3 cDNA and pT7-7 plasmid were separately reacted with two restriction enzymes, NdeI and HindIII. The vector and cDNA were then ligated at 16 °C overnight using T4 ligase in a reaction that included 7 μL of a mixture of T7-7 plasmid (0.5 μg) and cdb3 DNA (5 μg), 2 μL of 5× T4 ligase buffer (New England Biolab), and 1 μL T4 ligase (1 unit/μL). The crude ligase mixture containing the ligated plasmid (Fig. 1) was introduced into competent *E. coli* BL21 (DE3) cells, and the cells were proliferated in ampicillin containing LB growth medium (50 μg ampicillin/mL). After identification and amplification of ampicillin-resistant clones, the derived plasmids were reacted with PstI, ClaI, and NdeI and then analyzed in a 1.5% agarose gel. Clones containing the anticipated restriction fragments were selected for further study.

Selection of expression host and optimization of expression conditions

Escherichia coli BL21 (DE3), BL21 (DE3) (pLysS), and BL21 (DE3) (pLysE) are three host strains available in the pT7-7 expression system (Studier & Moffatt, 1986) that differ in the presence of a second plasmid containing T7 lysozyme and chloramphenicol resistance genes (pLysS and pLysE). T7 lysozyme is a natural inhibitor of the T7 RNA polymerase. The pLysE plasmid provides for a higher level of expression of T7 lysozyme than the pLysS plasmid. The saturated cell culture of the desired strain (containing 100 μg/mL ampicillin) was diluted 100 times into fresh ampicillin LB medium and shaken for another 2.5 h at 37 °C. Then, 1 mM (final) fresh IPTG with or without protease inhibitors (PMSF, benzamidine) and rifampicin were added. *Escherichia coli* cells were then aliquoted into several tubes for further studies of optimal temperature and induction times. From each aliquot, 0.9 mL of suspension was pelleted and lysed by adding 100 μL Laemmli gel sample buffer (Laemmli, 1970). Then, 15 μL of the supernatant was used for sodium dodecyl sulfate-polyacrylamide electrophoresis and Western blot analysis to evaluate the concentration and structural integrity of the expressed cdb3.

Optimal expression of cdb3 in E. coli BL21 (DE3) (pLysS)

Escherichia coli BL21 (DE3) (pLysS) containing the cdb3/pT7-7 vector was inoculated into 10 mL LB medium containing 100 μg/mL ampicillin and grown overnight at 37 °C. The saturated 10-mL culture was then used to inoculate a fresh 4 L of ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) supplemented LB medium. When cell density reached an optical density at 600 nm (OD_{600}) of 0.8–1.0, 1 mM fresh IPTG (final) was added. The culture was then transferred to room temperature (25 °C) for an additional 6 h of shaking. *Escherichia coli* was pelleted and resuspended in 50 mL of lysis buffer (20 mM Tris, pH 7.0, 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine, 0.2% β-mercaptoethanol, and 20 μg/mL PMSF) and then stored at −80 °C.

Purification of cdb3 from E. coli

The frozen *E. coli* pellets (see above) were thawed in a 37 °C water bath after addition of 50 mL lysis buffer containing 0.2% Triton. The bacteria were lysed by three freeze–thaw cycles consisting of immersion for 10 min in liquid N_2 followed by thawing in a 37 °C water bath and then sonication for 2 min at medium power with a Branson Sonifier. Cdb3 was collected by direct addition to the crude cell lysate of 20 mL DEAE-Sepharose CL-6B equilibrated with column buffer (10 mM phosphate buffer, pH 7.5, 1 mM EDTA, 0.2% β-mercaptoethanol, and 20 μg/mL PMSF). The mixture was allowed to mix on ice with occasional shaking for 2 h, and the DEAE-Sepharose CL-6B was then pelleted by low-speed centrif-

ugation (2,000 × *g*, 5 min). The supernatant was removed and the cellulose was washed three times with 200 mL of the same buffer. The washed DEAE-Sepharose CL-6B was then poured into a column (10 × 2.5 cm) and washed again with 50–100 mL column buffer containing 0.2 M NaCl until the OD_{280} approached zero. Most of the contaminating *E. coli* proteins were removed by this procedure. Cdb3 was then eluted with 200 mL of a salt gradient consisting of 0.2–0.5 M NaCl dissolved in column buffer. Cdb3 elutes from the column between 0.35 and 0.4 M NaCl, but its elution position is difficult to identify because of low molecular weight absorbing material. Therefore, the presence of cdb3 was verified by examining protein bands on a 12.5% mini polyacrylamide gel. The pooled fractions were concentrated by addition of $(NH_4)_2SO_4$ to 60% saturation (final), and the salt solution was stored at 4 °C overnight. Cdb3 was collected by centrifugation at 10,000 × *g* for 15 min, dissolved in 1 mL of phosphate-buffered saline (PBS: 150 mM NaCl, 5 mM phosphate buffer, pH 7.4, 0.2 mM dithiothreitol, 1 mM EDTA, and 20 μg/mL PMSF), and then applied to a PBS-equilibrated S-300 gel filtration column (117 × 2.7 cm). The first major elution peak of the Sephacryl S-300HR profile contained pure cdb3. The middle fractions of this initial peak were concentrated to 1–2 mL with a Diaflo apparatus from Amicon. Then, 30% glycerol (final) was added and cdb3 was stored at −20 °C. Under these conditions it was found to remain stable for at least 6 months.

Structural characterization of recombinant cdb3

Intrinsic fluorescence, circular dichroism, and Stokes radius studies using Sephacryl S-300 gel filtration chromatography were carried out as reported (Appell & Low, 1981; Low et al., 1984). Human erythrocyte cdb3 was purified from whole blood as previously described (Low et al., 1984). N-terminal blockage was evaluated by comparing the yields of derivatized amino acids from unmodified and deblocked recombinant cdb3 during Edman degradation (Aitken, 1990). Briefly, purified recombinant cdb3 was equally divided into two aliquots. One aliquot was subjected directly to Edman degradation, while the other was mildly hydrolyzed in methanol containing 0.5 M HCl for 48 h at 24 °C to remove any N-terminal blocking groups. The molar yield of each amino acid from each cycle of the Edman degradation of the two samples was then compared. Deblocking of N-blocked peptides by mild methanolic HCl hydrolysis should increase the yield of phenylthiohydantoin amino acids.

Functional characterization of recombinant cdb3

Analysis of the ability of recombinant cdb3 to inhibit aldolase was essentially the same as that previously reported (Low et al., 1987) with minor modifications. In brief, a substrate cocktail (0.1 mL of 12 mM fructose-1,6-bisphosphate plus 2 mL of 0.1 mM EDTA and 3.5 mM hydrazine sulfate, pH 6.0) was rapidly mixed with a 1-mL solution of aldolase and cdb3 prepared by mixing 2.2 μg aldolase with different amounts of cdb3. The OD_{240} was then continuously recorded for 5 min in an IBM UV–visible spectrophotometer, and the slope was used to calculate the residual aldolase activity. Ankyrin affinity for recombinant cdb3 was measured both by sedimentation analysis and competitive inhibition of ankyrin binding to stripped inside-out erythrocyte membrane vesicles (Thevenin & Low, 1990). For these assays, ankyrin was purified according to the method of Pinder et al. (1989) and radioiodinated with Bolton–Hunter reagent, as described elsewhere (Thevenin & Low, 1990).

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