Mapping of glycolytic enzyme-binding sites on human erythrocyte band 3

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Previous work has shown that GAPDH (glyceraldehyde-3-phosphate dehydrogenase), aldolase, PFK (phosphofructokinase), PK (pyruvate kinase) and LDH (lactate dehydrogenase) assemble into a GE (glycolytic enzyme) complex on the inner surface of the human erythrocyte membrane. In an effort to define the molecular architecture of this complex, we have undertaken to localize the binding sites of these enzymes more accurately. We report that: (i) a major aldolase-binding site on the erythrocyte membrane is located within N-terminal residues 1–23 of band 3 and that both consensus sequences D⁶DYED¹⁰ and E¹⁹EYED²³ are necessary to form a single enzyme-binding site; (ii) GAPDH has two tandem binding sites on band 3, located in residues 1–11 and residues

12–23 respectively; (iii) a PFK-binding site resides between residues 12 and 23 of band 3; (iv) no GEs bind to the third consensus sequence (residues D⁹⁰²EYDE⁹⁰⁶) at the C-terminus of band 3; and (v) the LDH- and PK-binding sites on the erythrocyte membrane do not reside on band 3. Taken together, these results argue that band 3 provides a nucleation site for the GE complex on the human erythrocyte membrane and that other components near band 3 must also participate in organizing the enzyme complex.

Key words: band 3–peripheral protein interaction, cytoplasmic domain of band 3, erythrocyte cytoskeleton, erythrocyte membrane structure, glycolysis, glycolytic enzyme complex.

INTRODUCTION

The GEs (glycolytic enzymes) GAPDH (glyceraldehyde-3-phosphate dehydrogenase), aldolase, PFK (phosphofructokinase), LDH (lactate dehydrogenase) and PK (pyruvate kinase) have been recently shown to assemble into a complex on the inner surface of the human erythrocyte membrane [1]. Thus each of the above enzymes was found to be membrane localized when intact erythrocytes were fixed and stained in their oxygenated states, but physiological stimuli that modulate glycolytic rates, such as deoxygenation and activation of tyrosine phosphorylation, were observed to shift the enzyme distribution into the cytosol. Further, antibodies that bind to the cdb3 (cytoplasmic domain of band 3) were seen to compete with each of the above enzymes and induce their displacement from the membrane into the cytosol. Taken together with results from others suggesting a pool of ATP on the membrane that can be filled by running glycolysis and emptied by running ion pumps [2], these observations argue for an organized assembly of membrane-bound GEs with functional significance.

Several lines of previous evidence have suggested that the GE docking site on the erythrocyte membrane might involve the N-terminus of band 3. First, as noted above, antibodies to the N-terminus of band 3 competitively displace the enzymes from the membrane [1]. Secondly, studies with purified fragments of band 3 demonstrate that the N-terminus of the polypeptide contains sequences that inhibit aldolase and GAPDH [3–6]. Thirdly, membrane-associated aldolase was found to be absent from erythrocytes of a patient with a mutant band 3 lacking the first 11 amino acids [7]. Fourthly, phosphorylation of band 3 on Tyr⁸ and Tyr²¹ is known to block binding of GAPDH, aldolase and PFK to band 3 *in vitro* [8,9] and to promote displacement of GEs from the membrane in intact cells [1]. Fifthly, resealing of a peptide comprising the N-terminus of band 3 into erythrocytes inhibits glycolysis, presumably due to enrichment of the cell with this GE-inhibitory sequence and incorporation of a monoclonal IgG against the same sequence leads to acceleration of glycolysis, presumably due to blockade of the same sequence [10].

Although the aforementioned results argue for a GE docking site on band 3 [11–15], multiple observations also exist to suggest that the acidic N-terminus of band 3 does not constitute the sole site of GE binding. Thus a peptide fragment comprising the N-terminus of band 3 inhibits aldolase and GAPDH with only 20 and 3 % of the potency of whole membranes respectively [5]. Further, although GEs are known to localize on the membranes of mouse and rat erythrocytes in a manner similar to that seen in humans (S. L. Alper, M. E. Campanella and P. S. Low, unpublished work), the N-terminal sequences of band 3 in the two species are not conserved. Thirdly, although the 65 N-terminal residues of band 3 are absent from kidney band 3 due to use of an alternate transcription start site, GAPDH is still localized on the membrane in kidney intercalated cells [16]. Finally, each band 3 monomer contains three distinct homologous sequences that correspond to GE-binding sites on other proteins [17,18], and only two of these sequences are in cdb3 (residues 1–379) [19]. Thus, besides the tandem homologous sequences D⁶DYED¹⁰ and E¹⁹EYED²³ at the extreme N-terminus, there is a third sequence (D⁹⁰²EYDE⁹⁰⁶) at the C-terminus of band 3 that might also be expected to bind GE.

In order to more precisely characterize the role of band 3 in organizing a GE complex on the membrane, we have expressed and purified multiple wild-type and mutant isoforms of both the N- and C-terminal domains of band 3 and have examined their associations with GEs. From these results, we have defined the sequences of band 3 that are critical for GAPDH, aldolase and PFK binding. We have also investigated the interactions of LDH and PK with band 3.

MATERIALS AND METHODS

Construction of truncated band 3 expression plasmids

To begin to define the binding sites of GEs on band 3, a series of truncation mutants of the cdb3 were prepared, including intact 43 kDa cdb3 with residues 1–11 deleted [del(1–11)], residues

Abbreviations used: BCA, bicinechonic acid; cdb3, cytoplasmic domain of band 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GE, glycolytic enzyme; GST, glutathione S-transferase; LDH, lactate dehydrogenase; mAb, monoclonal antibody; PFK, phosphofructokinase; PK, pyruvate kinase; Rh, Rhesus.

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Table 1 Primers for the band 3 constructs and site-directed mutagenesis

Introduced mutations and restriction enzyme sites are underlined. Introduced start codon and stop codon are written in boldface letters. Sn, sense; Asn, antisense; wt, wild type.

Mutant name	Template	Oligonucleotides
Thioredoxin-(1–55)	wt cdb3/pT7-7	Sn: 5'-ATGGAGGAGCTGCAGGATGA-3' Asn: 5'-GTGGGT TAC CCGGGTGTGATG-3'
Kidney	wt cdb3/pT7-7	Sn: 5'-CTCCAT ATG GACGAAAAGAACCCAGGAG-3' Asn: 5'-AAGCT TCAG AAGAGCTGGCCTGTCTGCT-3'
del(1–50)	wt cdb3/pT7-7	Sn: 5'-CTCCAT ATG CACCCGGGTACCCAGGAG-3' Asn: 5'-AAGCT TCAG AAGAGCTGGCCTGTCTGCT-3'
del(1–40)	wt cdb3/pT7-7	Sn: 5'-CTCCAT ATG GCAACAGCCACAGACTAC-3' Asn: 5'-AAGCT TCAG AAGAGCTGGCCTGTCTGCT-3'
del(1–31)	wt cdb3/pT7-7	Sn: 5'-CTCCAT ATG GAGGAGCCGGCAGCTCAC-3' Asn: 5'-AAGCT TCAG AAGAGCTGGCCTGTCTGCT-3'
del(1–23)	wt cdb3/pT7-7	Sn: 5'-CTCCAT ATG CCAGACATCCCCGAGTCC-3' Asn: 5'-AAGCT TCAG AAGAGCTGGCCTGTCTGCT-3'
del(1–11)	wt cdb3/pT7-7	Sn: 5'-CTCCAT ATG GAGGAGAATCTGGAGCAG-3' Asn: 5'-AAGCT TCAG AAGAGCTGGCCTGTCTGCT-3'
del(12–23)	del(1–11) cdb3/pT7-7	Sn: 5'-CTCCAT ATG GAGGAGCTGCAGGATGATTATGAAGACATGCCAGACATCCCCGAGTCC-3' Asn: 5'-AAGCT TCAG AAGAGCTGGCCTGTCTGCT-3'
Y8E	wt cdb3/pT7-7	5'-CCAGATTCTCCTCCATCATGTCTTCTCATCATCCTGCAGCTCCTCC-3'
Q ⁹ N ¹⁰	wt cdb3/pT7-7	5'-GATTCTCCTCCATCATGTTTGTATAATCATCTGCCAG-3'
Q ¹⁷ QQQ ²⁰	wt cdb3/pT7-7	5'-GGGATGCTGGGTCTTCATATTGCTGCTGCTGCAGATTCTCCTCCATCATGTC-3'
N ⁶ NYQN ¹⁰	wt cdb3/pT7-7	5'-CCATTCCATATGGAGGAGCTGCAGAATAATTATCAAAACATGATGGAG-3'
Q ¹⁹ QYQN ²³	wt cdb3/pT7-7	5'-AATCTGGAGCAGCAGCAATATCAAACCCAGACATCCCCGAGTCC-3'
Q ¹⁹ QAQN ²³	wt cdb3/pT7-7	5'-GAGAATCTGGAGCAGCAGCAAGCTCAAACCCAGACATCCCC-3'
N ⁶ NAQN ¹⁰ plus Q ¹⁹ QAQN ²³	Q ¹⁹ QAQN ²³ /pT7-7	5'-GAGGAGCTGCAGAATAATGCTCAAACATGATGGAGG-3'
GST-(872–911)	band 3/pDNA3	Sn: 5'-GGATCCGCTCTGCTGCCGCTCATC-3' Asn: 5'-AAGCT TCAC ACAGGCATGGCCACTTC-3'

12–23 deleted [del(12–23)], residues 1–23 deleted [del(1–23)], residues 1–31 deleted [del(1–31)], residues 1–40 deleted [del(1–40)], residues 1–50 deleted [del(1–50)] and residues 1–65 deleted (residues 66–379, kidney cdb3). For these constructs, the oligonucleotide primers shown in Table 1 were used in combination with wild-type cdb3 (residues 1–379) cDNA for PCR amplification. Sense primers were always designed to contain an NdeI site (underlined), followed by a start codon ATG (in boldface) and at least 18 bases encoding the corresponding residues of cdb3. To construct the residues 12–23 deletion mutant [del(12–23)], however, the del(1–23)/pT7-7 plasmid was used as a template, and nucleic acids encoding cdb3 residues 1–11 were simply incorporated into the sense primer. In all antisense primers, a HindIII site (underlined) followed by a stop codon TCA (in boldface) and at least 18 bases encoding the corresponding residues of cdb3 were used. Because the NdeI cleavage site in the sense strand was found to be too close to the end of the DNA fragment for efficient cleavage, the amplified DNA product was ligated into a pGEM-T easy vector (Promega), resulting in a cyclic cdb3/pGEM-T plasmid. The cdb3 fragment was then digested by NdeI and HindIII from the pGEM-T vector and ligated into the pT7-7 expression vector.

To construct the C-terminus band 3 (residues 872–911) expression plasmid, similar methods were used except that a BamHI site was added in a PCR sense primer, band 3 cDNA was used as the template and a pET21a-GST vector (where GST is glutathione S-transferase) was used as the expression vector to express the fusion protein, GST/C-terminus band 3 residues 872–911 [GST-(872–911)].

A pBAD-TOPO-TA expression kit (Invitrogen) was used to clone and express N-terminal residues 1–55 as a fusion protein with a thioredoxin and two hexahistidine (His₆) tags [thioredoxin-(1–55)], according to the manufacturer's instructions.

Finally, an expression plasmid for cdb3 lacking residues 29–52 [del(29–52)] was constructed by digesting wild-type (residues

1–379) cdb3/pT7-7 with *Ava*I and *Sma*I to release the nucleic acid fragment encoding residues 29–52. Single-stranded overhangs were then filled in by Klenow fragment polymerization and the two ends were ligated using T7 DNA ligase.

Site-directed mutagenesis

Site-directed mutagenesis was employed to construct all substitution mutants of cdb3. For this purpose, a QuikChange[®] mutagenesis kit (Stratagene) was used to introduce point mutations into the cdb3 coding region of the pT7-7 expression plasmid, largely according to the manufacturer's instructions. One primer was used to generate each mutant; introduced mutations are underlined, as shown in Table 1.

Expression, purification and characterization of mutated band 3

Before expression, all mutant cDNAs were confirmed by sequencing. All proteins were expressed in BL21(DE3) pLys S cells (Invitrogen) as described previously [20]. GST/C-terminus band 3 residues 872–911 [GST-(872–911)] was purified on a glutathione-Sepharose column (Amersham Biosciences). Thioredoxin/N-terminus residues 1–55 [thioredoxin-(1–55)] was purified with an Ni²⁺-affinity chromatography (Qiagen). Peptides including N-terminal residues 1–55 were cleaved from their thioredoxin tags with thrombin and purified on a Q-Sepharose FF (fast flow) column. Purification of other recombinant cdb3 domains and related mutants lacking His₆ or GST tags was performed as described previously [20,21]. Briefly, DEAE-Sepharose CL-6B column (Amersham Biosciences) chromatography of the crude cdb3 extract was followed by high-performance phenyl-Sepharose column (Amersham Biosciences) chromatography. SDS/PAGE and Western-blot analysis were then used to verify the purity of the resulting proteins. The pH-dependence of the intrinsic fluorescence of cdb3 was invariably employed to establish

that the cdb3 mutants underwent the same native conformational changes as wild-type cdb3 (residues 1–379) [22,23].

Aldolase assay and inhibitor-binding analysis

Increasing amounts of cloned wild-type cdb3 (residues 1–379) or its mutants were mixed in a cuvette with 32 pmol of rabbit muscle aldolase (Sigma) in a total volume of 0.1 ml, containing 10 mM sodium phosphate and 0.1 mM EDTA at pH 6.0. After a 5 min incubation, aldolase activity was measured at room temperature (25°C) in a final volume of 1 ml as described in [21] with minor modifications. Percentage inhibition of aldolase activity was calculated as follows:

$$\text{Inhibition (\%)} = (1 - A/B) \times 100$$

where *A* is activity in the presence of cdb3 and *B* is activity in the absence of cdb3.

GAPDH assay and inhibitor-binding analysis

Increasing amounts of cloned wild-type cdb3 (residues 1–379) or its mutants were mixed in a cuvette with 28 pmol of rabbit muscle GAPDH (Roche) in a total volume of 0.1 ml, containing 10 mM imidazole acetate, 0.1 mM EDTA, 0.5 mM sodium arsenate and 1 mM sodium phosphate (pH 7.0). After a 5 min incubation, GAPDH activity was measured in a final volume of 1 ml as described in [5] with minor modifications. Percentage inhibition of GAPDH activity was calculated as described above for aldolase.

PFK assay and inhibitor-binding analysis

Assay A

Increasing amounts of cloned wild-type cdb3 or its mutants were mixed with 16 pmol of rabbit muscle PFK (purified according to the procedure of Ling et al. [24]) in a total volume of 0.2 ml containing 10 mM Tris/HCl, 0.2 mM MgCl₂, 0.5 mM (NH₄)₂SO₄, 1 mM EDTA, 0.25 mM dithiothreitol and 1 mM ATP at pH 7.0. After a 5 min incubation at room temperature, 25 µl of 40 mM fructose-6-phosphate was added and the incubation was continued for precisely three additional minutes. The solution was then deproteinized with 1 ml of HClO₄ and the fructose-1,6-bisphosphate concentration was measured as described in [25].

Assay B

Increasing amounts of cloned cdb3 or its mutants were mixed with 16 pmol of rabbit muscle PFK as described above except that the total volume was 0.1 ml, and the buffer contained 10 mM Tris/HCl, 0.2 mM MgCl₂, 1 mM EDTA and 1 mM ATP (pH 7.0). After a 5 min incubation at room temperature, PFK activity was measured in a final volume of 1 ml as described in [6] with minor modification. Percentage PFK inhibition was calculated as described for aldolase.

LDH assay and inhibitor-binding analysis

Excess cdb3 or its mutants were incubated with rabbit muscle LDH (Sigma) in 10 mM sodium phosphate buffer (pH 7.5) for 5 min. LDH activity was then measured as described in [25] with minor modifications. The assay solution contained 10 mM sodium phosphate, 5 mM pyruvate and 0.2 mM NADH (pH 7.5) in a final volume of 1 ml.

PK assay and inhibitor-binding analysis

Excess cdb3 or its mutants were incubated for 5 min with rabbit muscle PK (Sigma) in 10 mM sodium phosphate (pH 7.5) and PK activity was measured as described in [25] with minor modifications. The assay solution contained 10 mM Tris/HCl, 2 mM MgCl₂, 5 mM KCl, 5 mM PEP (phosphoenolpyruvate), 1.5 mM NADH, 5 mM ATP and 1 mM LDH (pH 7.4) in a final volume of 1 ml.

GE competition assay

Increasing amounts of aldolase, PFK, LDH or PK were incubated for 5 min at room temperature with 1.7 µg of cdb3 in a total volume of 0.1 ml containing 10 mM imidazole acetate, 0.1 mM EDTA, 0.5 mM sodium arsenate and 1 mM sodium phosphate (pH 7.0). GAPDH was then added and the incubation was continued for 2 min. GAPDH activity was then measured in a total volume of 1 ml and compared with its activity under identical conditions in the absence of both GEs and cdb3 (taken as 100 %).

Measurement of direct GE binding to band 3

Purified wild-type (residues 1–379) or kidney (residues 66–379) cdb3 (0.1 µmol) was immobilized on to 1 ml Affi-Gel 15 beads (Bio-Rad) according to our previous methods [9]. Then, 25 µl of beads equilibrated in 10 mM imidazole buffer (pH 6.5) were then incubated with an excess of the desired pre-dialysed GE in a total volume of 0.4 ml. After 10 min incubation at room temperature with gentle agitation, the beads were washed three times with the same buffer and separated into two aliquots. One aliquot was processed for SDS/PAGE, while the other aliquot was stripped of bound GE in a total volume of 200 µl with 10 mM imidazole and 200 mM NaCl (pH 8.0). The eluted GE was then independently quantified by both measurement of its catalytic activity and determination of its protein concentration using the BCA (bicinchoninic acid) protein assay kit (Pierce).

RESULTS

To identify the protein domains of band 3 important for its interaction with GEs, different regions of band 3 or their truncation/substitution mutants were prepared as shown in Figure 1 and assayed for association with GEs. Because the binding of wild-type cdb3 to aldolase, GAPDH and PFK had been previously shown to inhibit each enzyme's catalytic activity and since inhibition assays have been widely used to study band 3 interaction with aldolase and GAPDH [3–6], our initial assays for enzyme binding were based on the reduction in each enzyme's catalytic activity upon addition of the desired construct of band 3.

Mapping of the aldolase-binding site on band 3

Although published results suggested that at least part of the membrane-binding site for aldolase might reside at the N-terminus of band 3 [4], the specific residues of band 3 involved in aldolase binding were never established. To identify these residues, wild-type cdb3 (residues 1–379), the cytoplasmically exposed residues of the C-terminus of band 3 (residues 872–911), kidney cdb3 (residues 66–379) and a peptide fragment of the N-terminus of band 3 (residues 1–55) were all compared for their abilities to inhibit aldolase catalysis. As shown in Figures 2(A) and 2(C), the peptide fragment comprising residues 1–55 of band 3 was found to reduce aldolase activity with nearly the same potency as intact cdb3. In contrast, kidney cdb3 (residues 66–379), which differs from wild-type cdb3 (residues 1–379) in that it lacks the first

Figure 1 Schematic representation of human cdb3 and its mutants used in the present study

Sequences that have been deleted in the different cdb3 mutants are represented as different boxes in the diagram. On the right of the panel is the name of each construct. For deletion mutants, the numbers in parentheses represent the deleted sequence. For substitution mutants, the substituted residues are written in boldface letters and underlined. GST-(872–911) denotes the C-terminal residues 872–911 of band 3 fused to GST at its N terminus.

65 amino acids, had no impact on aldolase activity. Furthermore, the C-terminus of band 3, which contains sequences similar to the extreme N-terminus of band 3, also caused no perturbation of aldolase function. Taken together, these results suggest that the aldolase-binding site on band 3 resides within the first 55 residues of the polypeptide.

To determine whether this aldolase-binding site might overlap the N-terminal amino acid on band 3, thioredoxin was fused to the N-terminus of the same 55 residue peptide [thioredoxin-(1–55)] that was shown above to have significant inhibitory potency and the resulting fusion protein was examined for aldolase inhibition. As shown in Figures 2(A) and 2(C), fusion of thioredoxin to the 55-residue peptide totally blocked the peptide's inhibitory potency, suggesting that the aldolase site overlaps and requires an unimpeded N-terminus of band 3.

To more precisely define the residues involved in aldolase binding, various deletion mutants of cdb3 were expressed and examined. As shown in Figures 2(A) and 2(D), aldolase activity was not inhibited by cdb3 constructs lacking residues 1–50, 1–40, 1–31 or 1–23, but was still suppressed by a cdb3 mutant lacking residues 29–52. These results suggest that the major residues involved in aldolase association are probably restricted to the first 23 amino acids of cdb3.

Because there are two homologous sequences within these initial 23 amino acids (i.e. residues M¹EELQDDYED¹⁰ and M¹²EENLEQEEYED²³), it seemed reasonable to postulate that the N-terminal 23-mer might contain two independent binding sites for aldolase. To test this hypothesis, we constructed cdb3 mutants that lacked either the first (residues 1–11) or second (residues 12–23) tandem repeat and examined each mutant's impact on aldolase activity. As shown in Figure 2(D), neither del(1–11) nor

del(12–23) cdb3 was capable of inhibiting aldolase. Therefore we conclude that both homologous sequences may be involved in a single aldolase interaction.

Finally, we have previously shown that phosphorylation of band 3 on Tyr⁸ and Tyr²¹ dissociates aldolase from band 3 [1,8,9], suggesting that these tyrosine residues are located in regions of the polypeptide that regulate aldolase association. In order to explore whether D⁶DYED¹⁰ and E¹⁹EYED²³ directly participate in aldolase binding, we mutated the residues to closely related counterparts (E → Q, D → N and Y → A) and examined the impact on aldolase inhibition. Importantly, neither complete substitution of all ten residues (N⁶NAQN¹⁰ plus Q¹⁹QAQN²³) nor replacement of only the first (N⁶NYQN¹⁰) or only the second (Q¹⁹QYQN²³) homologous sequence yielded a protein with any inhibitory potency (Figures 2B and 2E). However, single and double amino acid substitutions within these sequences generated mutants that retained some inhibitory potency. On the basis of these results, we conclude that the aldolase-binding site on band 3 resides within the N-terminal 23 amino acids and depends significantly on the presence of both homologous clusters of acidic residues for its potency.

Evaluation of the GAPDH-binding site on erythrocyte membrane band 3

An analogous catalytic inhibition assay was used to map the association of GAPDH with band 3. Briefly, the GAPDH-binding site was initially mapped to the N-terminal 55 amino acids of band 3 (Figures 3A and 3C) on the basis of the observations that (i) kidney cdb3 (residues 66–379) has no impact on GAPDH activity, (ii) the cytoplasmically exposed C-terminus of band 3 also

Figure 2 Effect of cdb3 and its mutants on aldolase activity

Increasing amounts of wild-type cdb3 or its mutants were mixed in a cuvette with 32 pmol of rabbit muscle aldolase (Sigma) in a total volume of 0.1 ml, containing 10 mM sodium phosphate and 0.1 mM EDTA, (pH 6.0). After 5 min incubation, a 0.9 ml solution containing 3.5 mM hydrazine sulfate, 0.1 mM EDTA and 100 μ g of fructose-1,6-bisphosphate (pH 6.0) was added. The absorbance at 240 nm was then monitored continuously for 5 min. Aldolase activity was calculated from the absorbance difference between the 100 and 300 s time points. (A) Effect of increasing concentrations of various truncation mutants of cdb3 on the percentage of aldolase inhibition. (B) Effect of increasing concentrations of various substitution mutants of cdb3 on the percentage of aldolase inhibition. (C–E) Inhibition of aldolase activity by cdb3 and its mutants at saturating cdb3 concentrations, the effect of wild-type cdb3 is taken as 100%. Maximal inhibition values were determined from the saturation curves in (A) and (B) and plotted for each cdb3 construct.

lacks inhibitory potency and (iii) the N-terminal 55 residue fragment of cdb3 replicates most of the intact protein's inhibiting capability. The GAPDH-binding site was then further narrowed by studies showing that the deletion mutant lacking only residues 29–52 [del(29–52)] as well as all other constructs containing residues 1–23 displayed inhibitory potencies similar to wild-type cdb3, whereas all truncation mutants lacking residues 1–23 [i.e. kidney cdb3 (residues 66–379), del(1–50), del(1–40), del(1–31) and del(1–23)] show no effect on GAPDH activity (Figures 3A and 3D). Further mutagenesis experiments demonstrate that the tandem acidic clusters described above (i.e. D⁶DYED¹⁰ and E¹⁹EYED²³) are also required for GAPDH binding, since the corresponding uncharged substitution mutant (i.e. N⁶NAQN¹⁰ plus Q¹⁹QAQN²³) displays no inhibitory potency (Figures 3B and 3E).

More detailed analyses, however, demonstrated that the specific residues required for GAPDH binding are distinct from those required for aldolase inhibition. First, the large thioredoxin cap at the N-terminus of residues 1–55 [thioredoxin-(1–55)] that completely blocked aldolase association was found to have no effect on the GAPDH interaction (Figure 3C; compare with Figure 2C). Secondly, deletion of residues 1–11 of cdb3 [del(1–11)] caused only marginal loss of GAPDH inhibitory potency, even though it totally abrogated aldolase inhibition. Thirdly, deletion of residues 12–23 of cdb3 [del(12–23)] yielded a mutant that still inhibited GAPDH (Figure 3D; compare with Figure 2D) but did not affect aldolase. And finally, mutation of only one of the two acidic clusters at the N-terminus of cdb3 (either D⁶DYED¹⁰ to

N⁶NYQN¹⁰ or E¹⁹EYED²³ to Q¹⁹QYQN²³) generated a construct with most of wild-type cdb3's inhibitory potency towards GAPDH (Figure 3E), even though it completely eliminated an association with aldolase (Figure 3E; compare with Figure 2E). On the basis of these observations, we conclude that the GAPDH-binding site (i) resides primarily within the N-terminal 23 residues of band 3, (ii) does not overlap the N-terminal amino acid and (iii) probably involves two independent binding sites (residues 1–11 and 12–23), each of which can inhibit by itself, but both of which must be present to achieve complete inhibition.

Evaluation of the PFK-binding site on erythrocyte membrane band 3

Although no attempt has been reported to map the PFK docking site on band 3, previous work has shown that PFK does interact with erythrocyte membranes [6,26] and its activity can be inhibited by addition of cdb3 [6]. As with aldolase and GAPDH, the PFK-binding site on band 3 was mapped by identifying the residues of band 3 required for inhibition of the enzyme (see the Materials and methods section). As shown in Figure 4, PFK activity is not affected by the presence of either kidney cdb3 (residues 66–379) or the C-terminus of intact band 3, implicating the N-terminus of band 3 as the likely binding site for PFK. Further, all constructs of cdb3 containing residues 12–23 [i.e. wild-type cdb3 (residues 1–379), del(1–11) and the peptide fragments containing residues 1–55] are observed to inhibit PFK catalysis, whereas all cdb3 mutants lacking residues 12–23 [i.e. kidney del(1–65) cdb3, del(1–23) and del(12–23)] exert no effect on PFK

Figure 3 Effect of cdb3 and its mutants on GAPDH activity

Increasing amounts of wild-type cdb3 and its mutants were mixed in a cuvette with 28 pmol of rabbit muscle GAPDH (Roche) in a total volume of 0.1 ml containing 10 mM imidazole acetate, 0.1 mM EDTA, 0.5 mM sodium arsenate and 1 mM sodium phosphate (pH 7.0). After a 5 min incubation, 0.9 ml of the same imidazole buffer containing 250 μ g of NAD⁺ and 5 μ g of glyceraldehyde-3-phosphate was added and the absorbance at 340 nm was monitored continuously for 3 min. GAPDH activity was calculated from the absorbance difference between the 0 and 50 s time points. (A) Effect of increasing concentrations of various truncation mutants of cdb3. (B) Effect of increasing concentrations of various substitution mutants of cdb3. (C–E) Inhibition of GAPDH activity by cdb3 and its mutants at saturating cdb3 concentrations, the effect of wild-type cdb3 is taken as 100%. Saturation curves shown in (A) and (B) were determined and the maximal inhibition at saturating cdb3 concentration was plotted for each cdb3 construct.

activity. Taken together with results showing that the fusion protein, thioredoxin-(1–55), displays the same inhibitory potency as the corresponding unobstructed peptide (i.e. residues 1–55), it can be concluded that the PFK-binding site is more distal from the N-terminus of cdb3 than the aldolase-binding site, but resides between residues 12 and 23 of band 3.

Evaluation of the LDH- and PK-binding sites on erythrocyte membrane band 3

Because LDH and PK can be displaced from the membrane by a mAb (monoclonal antibody) against cdb3 [1], we naturally anticipated that LDH and PK would also associate with band 3. Further, because the activities of aldolase, GAPDH and PFK are strongly inhibited by cdb3, we began our investigation of these interactions by examining whether the catalytic activities of LDH and PK might be similarly inhibited by cdb3. As shown in Table 2, neither LDH nor PK catalysis is perturbed in any detectable manner upon addition of cdb3, suggesting that if either enzyme was to bind cdb3, the interaction must not be inhibitory.

To explore whether a non-inhibitory association of LDH or PK with cdb3 might occur, we first designed a competition assay to examine whether LDH or PK might block association of GAPDH with its inhibitory site on cdb3. For this purpose, cdb3 was incubated with various GEs (aldolase, PFK, LDH or PK), after which GAPDH was added and its catalytic activity was measured. As anticipated from the above mapping studies and previous work from von Ruckmann and Schubert [27], both aldolase and PFK reduced cdb3's inhibition of GAPDH (Figures 5A and 5B), confirming their overlapping binding sites on cdb3. However,

addition of neither LDH nor PK was found to have any impact on inhibition of GAPDH by cdb3 (Figures 5C and 5D), indicating that neither enzyme docks at the classical GE-binding site on the N-terminus of band 3.

To more directly measure association of LDH and PK with cdb3, wild-type (residues 1–379) and kidney cdb3 (residues 66–379) were immobilized on Affi-Gel 15 beads and incubated with excess GEs in 10 mM imidazole buffer. After washing, bound GEs were eluted from the immobilized cdb3 with 200 mM NaCl (pH 8.0) (i.e. conditions known to elute all GEs from the erythrocyte membrane [5]), and the activities of the various GEs in the eluent were measured. As shown in Figure 6(A), neither aldolase nor GAPDH was seen to bind the immobilized kidney cdb3 (residues 66–379), even though both enzymes readily copelleted with wild-type cdb3 (residues 1–379). These results confirm that aldolase and GAPDH indeed bind wild-type (residues 1–379) but not kidney cdb3 (residues 66–379). More importantly, neither LDH nor PK was found to associate with the immobilized wild-type cdb3 (residues 1–379), since neither enzyme activity could be detected in the high-salt eluent. Further, to ensure that neither LDH nor PK had bound to the immobilized cdb3 but failed to elute, SDS sample buffer was added to beads, and after boiling, the supernatants were loaded directly on to SDS/polyacrylamide gels for released GEs. Once again, although binding of GAPDH and aldolase was easily measured by this assay, no association of either LDH or PK could be detected under the same conditions (Figure 6B). We therefore conclude that neither LDH nor PK associates with cdb3.

Finally, because the crystal structure of cdb3 demonstrates that the N-terminus of band 3 resides next to the site where the

Figure 4 Effect of cdb3 and its mutants on PFK activity

(A) Increasing amounts of wild-type cdb3 and its mutants were incubated with 16 pmol of PFK in a total volume of 0.2 ml including 10 mM Tris/HCl, 0.2 mM MgCl₂, 0.5 mM (NH₄)₂SO₄, 1 mM EDTA, 0.25 mM dithiothreitol and 1.2 mM ATP at pH 7.0; 25 μ l of fructose-6-phosphate at 40 mM was then added and the solution was incubated for precisely 3 min. HClO₄ (1 ml) was added to deproteinize the solution, and after centrifugation, the supernatant was adjusted to pH 3.5 by addition of potassium carbonate solution. After 15 min on ice, 0.5 ml of supernatant was mixed with 0.5 ml of 10 mM sodium phosphate buffer (pH 7.0) and then NADH plus an enzyme mixture (aldolase, α -glycero-phosphate dehydrogenase and triose-phosphate isomerase) were added. The rate of change in absorbance at 340 nm was monitored with time and used to calculate PFK activity. Both assay A and assay B (as mentioned in the Materials and methods section) give similar results. (B) Inhibition of PFK activity by cdb3 and its mutants at saturating concentrations, the effect of wild-type cdb3 is taken as 100%. Saturation curves shown in (A) were determined and the maximal inhibition at saturating cdb3 concentration was plotted for each cdb3 construct.

polypeptide enters the membrane [28], it seemed reasonable to posit that the aforementioned ability of a mAb against cdb3 to displace LDH and PK from the membrane might arise from its ability to dislodge LDH and PK from a proximal sequence in the membrane-spanning domain of band 3. Indeed, since aldolase, GAPDH and PFK are known to bind to acidic sequences (D⁶DYED¹⁰ and E¹⁹EYED²³) in cdb3, it was anticipated that the homologous sequence D⁹⁰²EYDE⁹⁰⁶ at the C-terminus might constitute the unidentified binding site for LDH and PK. However, as shown in Table 2, a peptide comprising the exposed C-terminus of band 3 exerted no impact on the catalytic activity of either enzyme. Further, direct examination of the association of the C-terminus of band 3 with GEs using a GST fusion protein of C-terminal residues 872–911 immobilized on glutathione beads showed that none

Table 2 Effect of saturating concentrations of cdb3 and its mutants on the catalytic activities of various GEs

Band 3 construct	GAPDH activity (%)	Aldolase activity (%)	PFK activity (%)	LDH activity (%)	PK activity (%)
No band 3	100	100	100	100	100
Wild-type cdb3 (1–379)	7 $\pm$ 1.8	35 $\pm$ 0.5	21 $\pm$ 0.7	102 $\pm$ 4.3	107 $\pm$ 8.7
Kidney cdb3 (66–379)	100 $\pm$ 4.6	100 $\pm$ 4.3	98 $\pm$ 0.2	98 $\pm$ 7.3	106 $\pm$ 7.1
Thioredoxin-(1–55)	22 $\pm$ 3.2	95 $\pm$ 3.3	18 $\pm$ 1.7		
N-terminal residues 1–55 (1–55)	21 $\pm$ 1.7	47 $\pm$ 0.1	22 $\pm$ 3.6		
del(1–23) cdb3	93 $\pm$ 3.1	100 $\pm$ 1.9	96 $\pm$ 9.3		
del(1–11) cdb3	38 $\pm$ 0.1	92 $\pm$ 6.8	33 $\pm$ 3.7		
del(12–23) cdb3	75 $\pm$ 1.5	102 $\pm$ 0.6	94 $\pm$ 6.5		
GST-(872–911)	105 $\pm$ 7.9	99 $\pm$ 2.9	92 $\pm$ 8.3	96 $\pm$ 4.1	97 $\pm$ 7.7

Figure 5 Comparison of the inhibition of GAPDH by cdb3 in the absence and presence of increasing concentrations of other GEs

Increasing amounts of aldolase, PFK, LDH, or PK were incubated for 5 min with 40 pmol of cdb3 in a total volume of 0.1 ml, containing 10 mM imidazole acetate, 0.1 mM EDTA, 0.5 mM sodium arsenate and 1 mM sodium phosphate (pH 7.0). GAPDH was added and incubated for an additional 5 min before GAPDH activity was measured in a final volume of 1 ml as described above. GAPDH activity in the absence of cdb3 was taken as 100%. (A) Inhibition of GAPDH by cdb3 in the presence of aldolase. (B) Inhibition of GAPDH by cdb3 in the presence of PFK. (C) Inhibition of GAPDH by cdb3 in the presence of LDH. (D) Inhibition of GAPDH by cdb3 in the presence of PK.

of the GEs co-pelleted with the construct (results not shown), although a similar construct has been shown to bind carbonic anhydrase II [29]. On the basis of these results, we conclude that the binding sites for LDH and PK on the human erythrocyte membrane do not reside on a major cytoplasmically exposed domain of band 3.

Figure 6 Comparison of direct binding of GEs to wild-type cdb3 and kidney cdb3

Purified wild-type (residues 1–379) or kidney (residues 66–379) cdb3 (0.1 μ mol) was immobilized on 1 ml of Affi-Gel 15 beads. To 25 μ l of immobilized cdb3 equilibrated in 10 mM imidazole buffer (pH 6.5), excess GE (dialysed against the same buffer) was added and incubated for 10 min at room temperature under gentle agitation. The beads were washed three times with the same buffer and separated into two aliquots. One aliquot was stripped of bound GE in a total volume of 200 μ l with 10 mM imidazole and 200 mM NaCl (pH 8.0) and the eluted GE concentration was quantified both by measurement of its catalytic activity and determination of its protein concentration using the BCA assay (A). (Analysis of the samples by BCA assay yielded results similar to the activity data and so the results are not shown.) Alternatively, SDS sample buffer was added to the beads, and after boiling, supernatants were loaded directly on to SDS/polyacrylamide gels for analysis of GEs. wt, GEs released from wild-type cdb3; kidney, GEs released from kidney cdb3; control, pure GEs loaded directly on to gel as molecular-mass marker (B).

Figure 7 Diagram showing residues at the N-terminus of band 3 that participate in GE binding

A possible consensus binding sequence is highlighted in boldface italicized letters. For GAPDH, the boldface box defines the dominant binding site, while the lighter box indicates a weaker interaction site.

DISCUSSION

In the present study, we have provided evidence that aldolase, GAPDH and PFK occupy distinct but overlapping binding sites within the first 23 amino acids of band 3. Thus, although all three enzymes compete for occupancy of this same peptide on band 3, deletion of residues 1–11 eliminates aldolase inhibition, moderately reduces GAPDH inhibition, but has no effect on PFK inhibition [Table 2; compare wild-type cdb3 with del(1–11) cdb3]. Further, fusion of thioredoxin to the N-terminus of band 3 totally abrogates aldolase binding, but has little effect on GAPDH or PFK binding [Table 2; compare N-terminal residues 1–55 (1–55) with thioredoxin/N-terminal residues 1–55 [thioredoxin-(1–55)]}. Because inhibition of aldolase catalysis is also eliminated by deletion of residues 12–23, we hypothesize that aldolase requires the entire N-terminal 23 residues of band 3, including both acidic sequences (D⁶DYED¹⁰ and E¹⁹EYED²³), to form a single association site. Although GAPDH binding also occurs within this N-terminal sequence, there may exist two GAPDH docking sites on the peptide, since deletion of either residues 1–11 or 12–23 still yields a polypeptide with much of its initial inhibitory potency. In contrast, the PFK-association site must localize largely to residues 12–23, since deletion of residues 1–11 has little impact on its binding. These conclusions are summarized in Figure 7.

When Steck and co-workers [4] found that a 23 kDa N-terminal fragment of band 3 exhibited only 20 % of the affinity for aldolase of intact band 3, they assumed that the fragment's conformation became altered during cleavage and isolation from erythrocytes.

Although we have found that human erythrocyte cdb3 expressed in *Escherichia coli* has a similar structure and peripheral protein-binding ability as band 3 isolated from fresh erythrocytes [20], we have also found that the 55-residue N-terminal fragment of band 3 exhibits reduced affinity for aldolase and GAPDH compared with intact cdb3 (Table 2). An alternative explanation of these discrepancies is that minor subsite interactions occur with other regions of band 3 and that these subsites were not identified in our mapping studies.

Recognizing that at least three GEs compete for the same sequence on band 3, and knowing that GE occupancy of this site is blocked by ankyrin [30], the question naturally arises whether there are sufficient copies of band 3 to simultaneously bind ankyrin and each of the above GEs. Published results indicate that there are approx. 120 000 copies of ankyrin, approx. 500 000 copies of GAPDH, approx. 20 000 copies of aldolase and approx. 6000 copies of PFK per cell [31,32]. Given there are 1 200 000 monomers of band 3 per erythrocyte [32], we conclude that there are more than enough band 3s to attach all of the aforementioned peripheral proteins to the membrane.

Although LDH and PK are organized into complexes with aldolase, GAPDH and PFK at the inner surface of the human erythrocyte membrane [1], neither LDH nor PK was found to associate directly with band 3. This observation was unexpected, but it can nevertheless be explained by one of two possibilities: (i) GEs that directly bind band 3, such as aldolase, GAPDH and PFK, serve as a scaffold on to which LDH and PK assemble or (ii) another protein that interacts with band 3 provides the membrane docking sites for LDH and PK. While results showing that (i) binding of anti-cdb3 antibodies to cdb3, (ii) association of deoxyhaemoglobin with cdb3, or (iii) phosphorylation of cdb3 on Tyr⁸ and Tyr²¹ displace LDH, PK, GAPDH, PFK and aldolase from the erythrocyte membrane in intact cells are consistent with either interpretation [1], a recent observation that Rh (Rhesus) protein interacts with PK [33] would seem to favour the latter explanation. Thus Rh proteins are known to associate tightly with band 3 in a complex of membrane-spanning proteins that also includes glycophorin A, CD47 and perhaps other proteins [34], and it would not be unreasonable to postulate that Rh and band 3 collectively provide a framework on to which GEs can assemble. A single band 3 tetramer and one Rh dimer could conceivably organize up to six GEs in one location.

The observation that GAPDH, aldolase and PFK are all inhibited upon binding band 3 raises a perplexing conundrum: if GEs are organized into a complex on band 3 in order to generate ATP for local consumption (e.g. to run adjacent ion pumps) [2], then why should the interaction with band 3 be inhibitory? While we cannot provide a definitive answer to this question, three possible explanations arise. First, the complex of GEs that generates the membrane pool of ATP [2] could be distinct from the band 3 complex described here and elsewhere [1,4,5]. Secondly, regulatory stimuli that accelerate glycolysis could reorganize the complex into a non-inhibitory conformation. Thirdly, since each of the GEs retains some catalytic activity upon binding band 3, this residual activity could be sufficient to supply the energy needs of associated ion pumps. In this latter scenario, the major function of the less active enzymes in the complex could be structural, i.e. formation of a membrane compartment that could funnel ATP directly to ion pumps. Obviously, considerable research will be required before the function of the GE complex on band 3 can be elucidated.

Finally, the fact that the homologous acidic sequences at the N-terminus of band 3 (i.e. D⁶DYED¹⁰ and E¹⁹EYED²³) appear to constitute important determinants of GE binding raises the question whether these sequences might constitute consensus

GE-binding sites in non-erythroid cells as well. Fortunately, a number of non-erythroid proteins have already been reported in the literature to bind GEs, allowing examination of their sequences for the postulated consensus GE-binding site. The most studied GE-binding proteins in the literature are tubulin [17], GLUT4 [18], Wiskott–Aldrich syndrome protein [35], F-actin [36,37], myocilin [38] and Rab2 [39]. Significantly, homology searches of these proteins reveal that four of these proteins contain a sequence similar to that found in band 3. Thus the primary structures of F-actin, α -tubulin, GLUT4 and myocilin contain the sequences E³⁶¹YDE³⁶⁴, E⁴²⁹KDYEE⁴³⁴, E⁴⁹⁹LEY⁵⁰² and E²³⁰YD²³² respectively. Although GE binding has never been reported for troponin T, we have also recently noted a similar sequence in its primary structure (E⁵EQEYEE¹²) and have explored whether it too might bind GEs. Preliminary results demonstrate that troponin T indeed binds and inhibits aldolase, GAPDH and PFK with a potency similar to that of cdb3 (K. Simon and P. S. Low, unpublished work). On the basis of these observations, we hypothesize that acidic amino acids on either side of a tyrosine residue constitute a consensus sequence for GE binding [17]. Nevertheless, caution must be exercised in over-interpreting such sequence information, since a similar sequence (D⁹⁰²EYDE⁹⁰⁶) is also found at the C-terminus of band 3, and to date we have been unable to identify any GE that can bind to this site.

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