Tissue-Specific Alternative Splicing of Protein 4.1 Inserts an Exon Necessary for Formation of the Ternary Complex With Erythrocyte Spectrin and F-Actin

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Erythrocyte protein 4.1 is a major constitutive element of the erythrocyte membrane skeleton and is present in many diverse nonerythroid tissues as well. Its role was first defined in the erythrocyte, in which it exists as an 80-Kd phosphoprotein that is critical to the integrity of the red blood cell membrane skeleton. Protein 4.1 forms a ternary complex with erythrocyte spectrin and actin filament fragments, and thereby amplifies the interaction of the latter two proteins. The other major components of the erythrocyte membrane skeleton are protein 4.9 and adducin. The spectrin-based cytoskeleton is linked to the transmembrane glycoproteins and lipid bilayer through protein 4.1’s interaction with integral membrane proteins glycoporphin A and C and band 3. As well as through ankyrin’s interaction with band 3, protein 4.1 also binds to myosin and to phosphatidyserine. Earlier investigations have shown that the spectrin/actin-binding site on erythrocyte protein 4.1 is located within a 65 amino acid fragment produced by mild chymotryptic digestion of the intact protein. Further attempts to define the binding site, using antibodies specific for sequences near the N- and C-termini of the 10-Kd fragment, suggested that structural features throughout the sequence may be involved in the formation of the spectrin/actin/protein 4.1 complex. The present study was undertaken to more precisely define the structural features of erythrocyte protein 4.1 that are required for formation of the ternary complex with spectrin and F-actin.

Nonerythroid homologs of protein 4.1 have been described in many types of cells, including fibroblasts, endothelial cells, neurons, Sertoli cells, granulocytes, lymphocytes, and platelets. These isoforms exhibit considerable structural and functional diversity. Although the functions of the nonerythroid protein 4.1 isoforms have not been well defined, they are apparently not strictly confined to the cell membrane. For example, immunofluorescence studies indicate that protein colocalizes with stress fibers in fibroblasts, endothelial cells, and Sertoli cells, and occurs in perinuclear regions and at areas of cell-cell contact in endothelial cells. A larger (135 Kd) isoform of protein 4.1, predominant in lymphocytes, is associated with nuclear structures in cultured canine kidney epithelial cells.

Considerable effort has been focused on characterizing the structures, functions, and genetic regulation of the isoforms of protein 4.1. We and others have reported that the sequences of cDNA clones isolated from human reticulocyte and T-cell leukemia (MOLT-4) libraries exhibit differential expression of at least 10 nucleotide sequence motifs. One of these sequences is expressed in late stage erythroid cells, but not in more immature erythroid cells or lymphocytic cells. This sequence, designated as motif I, encodes a 21 amino acid sequence found at the N-terminus of the chymotryptic peptide that contains the spectrin/actin binding site. The presence of motif I within this peptide and the fact that expression of motif I appears to be characteristic of late erythroid development led us to investigate the role of this sequence in the formation of the ternary complex of protein 4.1, F-actin, and erythrocyte spectrin that is critically important for the maintenance of normal erythrocyte shape and resistance of the cell to fragmentation by the shear forces encountered as the cell circulates through the vascular system. We report here that the amino acid sequence encoded by motif I is necessary for the efficient formation of the ternary complex. This suggests that the association of protein 4.1 with spectrin and actin in
FUNCTIONAL PROPERTIES OF PROTEIN 4.1 ISOFORMS

A model of erythrocyte protein 4.1, indicating the chymotrypsin-sensitive sites, the spectrin/actin-binding domain, and the location of the sequence encoded by motif I. (B) Plasmids pEry2 and pEry12. Models of the coding sequences of plasmids used for transcription of the recombinant protein 4.1 isoforms lacking and containing motif I, respectively, showing the relative positions and presence or absence of motifs I, II, and III. The plasmids were generated as described in Materials and Methods.

this complex is a specialized function of the erythroid isoform. A preliminary report has been presented in abstract form.

MATERIALS AND METHODS

Antibodies. Three polyclonal antisera were generated and affinity-purified as previously described. Anti-4.1 recognizes epitopes distributed throughout erythrocyte protein 4.1. Anti-10a and anti-10b are specific for epitopes within the region encoded by motif I and the C-terminal region of the 10-Kd peptide, respectively.

Construction of cDNA clones suitable for transcription into mRNA. Specific protein 4.1 cDNA clones pFL7.13 and pEry2 have been previously isolated and sequenced. pFL7.13, which contains both motif I and motif II, was isolated from a human fetal liver (erythroid) cDNA library. pEry2, which contains motif II but lacks motif I, was constructed as previously described. A new recombinant clone, pEry12, which contains both motif I and motif II, was constructed by removing the fragment containing motif I and motif II from pFL7.13 with restriction enzymes Stu I and Sac I and inserting the isolated fragment into the homologous site in pEry2 from which the Stu I/Sac I fragment that lacked motif I had been removed. The nucleotide sequences of pEry2 and pEry12 were confirmed by DNA sequence analysis as described elsewhere; they are identical except for the 63 nucleotide sequence of motif I that is present in the pEry12 plasmid but absent from pEry2.

In vitro transcription and translation. Synthetic capped “sense” strand mRNAs were generated from the SP6 promoter within the pEry2 and pEry12 plasmids as previously described. The sense mRNAs were translated in a rabbit reticulocyte system (Promega, Madison, WI), using [35S]-methionine to radiolabel the newly synthesized proteins. The following components were added in order: 1 to 2 μg synthetic mRNA (preheated to 70°C for 5 minutes to denature the mRNA secondary structure), 50 U RNasin ribonuclease inhibitor, 6 μL of 1 mmol/L amino acid mixture without methionine (Promega), 50 μL rabbit reticulocyte lysate (Promega), and 8 μL L-[35S]-methionine (Amersham, Arlington Heights, IL; >1,000 Ci/mmol). The volume was brought to 100 μL with diethylpyrocarbonate-treated water, and the synthesis was performed at 37°C for 1 hour.

Preparation of recombinant 10-Kd peptides. The recombinant 10-Kd protein 4.1 peptides were prepared by the use of the pGEX expression vector pGEX-2T (Pharmacia-LKB, Piscataway, NJ). The constructs were prepared with a 201-bp insert corresponding to nucleotides 2014 through 2214, or a 138-bp insert corresponding to nucleotides 2080 through 2214, containing or lacking the 63-bp motif I, respectively. Escherichia coli strain DH5α (GIBCO-BRL, Gaithersburg, MD) were transformed with the recombinant pGEX plasmid as described. The lysate buffer contained the protease inhibitors phenylmethylsulfonyl fluoride (PMSF; 50 μg/mL), leupeptin (3.4 μg/mL), pepstatin (8 μg/mL), benzamidine (200 μg/mL), aprotinin (4 μg/mL), chymostatin (8 μg/mL), and D-amino caproic acid (13 μg/mL). The glutathione-agarose beads bearing the glutathione S-transferase/10-Kd peptide fusion proteins were washed multiple times by batch centrifugation to remove contaminating proteins and the protease inhibitors. Cleavage of the fusion proteins to yield the recombinant peptides was performed by the addition of thrombin to the washed beads in a thrombin:peptide ratio of 1:1,000 (wt/wt). Diisopropylfluorophosphate (1 mmol/L) was then added to inhibit the thrombin.

Formation of the ternary complex of protein 4.1, spectrin, and actin. Human erythrocyte spectrin and protein 4.1 and rabbit muscle actin were prepared, and the formation of the ternary complex was assayed as previously described. When assaying the formation of the complex with recombinant protein 4.1 isoforms generated by in vitro synthesis using the reticulocyte lysate system, the mixtures containing the in vitro synthesized proteins were diluted with 2 vol of incubation buffer (130 mmol/L KCl, 20 mmol/L NaCl, 10 mmol/L Tris, 0.1 mmol/L EDTA, 0.5 mmol/L MgCl2, 0.2 mmol/L ATP) and then on ice for 30 minutes and then on ice for 60 minutes. One hundred microliters of the samples was then layered onto 50-μL cushions of 20% sucrose in 10-Kd protein 4.1 peptides. The recombinant 10-Kd peptides were prepared by the use of the pGEX expression vector pGEX-2T (Pharmacia-LKB, Piscataway, NJ). The constructs were prepared with a 201-bp insert corresponding to nucleotides 2014 through 2214, or a 138-bp insert corresponding to nucleotides 2080 through 2214, containing or lacking the 63-bp motif I, respectively. Escherichia coli strain DH5α (GIBCO-BRL, Gaithersburg, MD) were transformed with the recombinant pGEX plasmid as described. The lysate buffer contained the protease inhibitors phenylmethylsulfonyl fluoride (PMSF; 50 μg/mL), leupeptin (3.4 μg/mL), pepstatin (8 μg/mL), benzamidine (200 μg/mL), aprotinin (4 μg/mL), chymostatin (8 μg/mL), and D-amino caproic acid (13 μg/mL). The glutathione-agarose beads bearing the glutathione S-transferase/10-Kd peptide fusion proteins were washed multiple times by batch centrifugation to remove contaminating proteins and the protease inhibitors. Cleavage of the fusion proteins to yield the recombinant peptides was performed by the addition of thrombin to the washed beads in a thrombin:peptide ratio of 1:1,000 (wt/wt). Diisopropylfluorophosphate (1 mmol/L) was then added to inhibit the thrombin.

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Formation of the ternary complex of 10-Kd peptide, spectrin, and actin. When assaying the incorporation of the 10-Kd protein 4.1 peptides in the ternary complex, the incubation mixtures contained 2 μg peptide, 15 μg F-actin, and 10 μg spectrin dimer in a final volume of 150 μL. The incubation buffer consisted of 3 mmol/L sodium phosphate, 10 mmol/L Tris, 130 mmol/L KCl, 20 mmol/L NaCl, 2 mmol/L MgCl2, 0.2 mmol/L ATP, pH 7.5. In the competition experiments, 1.6, 4, 8, 16, 40, and 80 μg of protein 4.1 (0.1-, 0.25-, 0.5-, 1.0-, 2.5-, and 5.0-fold molar excess over peptide, respect...
tively) were included in the incubation mixture. Control samples included binary mixtures of peptide and spectrin, peptide and actin, and the peptide alone. The samples were maintained at room temperature for 90 minutes and then centrifuged at 100,000g for 1 hour in a Type 42.2Ti rotor (Beckman). Supernatants and pellets were analysed by SDS-PAGE and Western blotting using polyclonal rabbit anti-10a and anti-10b antibodies. Immunoreactive bands were visualized using horseradish peroxidase–conjugated donkey antirabbit IgG and enhanced chemiluminescence (Amersham).

RESULTS

Characterization of the recombinant protein isoforms. To examine the possible role of the sequence encoded by motif I in the formation of the protein 4.1–actin–spectrin ternary complex, recombinant forms of both the 80-Kd protein 4.1 and the 10-Kd spectrin/actin-binding fragment that differed only in the sequence encoded by motif I were generated. Isoforms of protein 4.1 were synthesized by in vitro translation of mRNAs transcribed from specific protein 4.1 cDNA clones, pEry2 and pEry12, as described in Materials and Methods. The protein transcribed from pEry12 mRNA is recognized by two polyclonal antisera, one recognizing epitopes distributed throughout erythrocyte protein 4.1 (anti-4.1) and the other specific for epitopes within the region coded by motif I (anti-10a), whereas the protein transcribed from pEry2 is recognized only by anti-4.1 (Fig 2).

Formation of ternary complexes of recombinant protein 4.1 isoforms with erythrocyte spectrin and actin. The [35S]-labeled protein 4.1 isoforms with (+) and without (−) motif I were incubated with spectrin alone, actin alone, or spectrin and actin together, and centrifuged to pellet any spectrin-actin-protein 4.1 complexes that formed, as described in Materials and Methods. The supernatants and pellets were subjected to SDS-PAGE and the gels were dried and autoradiographed. In the presence of both spectrin and actin, the isoform containing motif I sedimented with F-actin and spectrin, in contrast to the results with the isoform that lacked motif I, which remained in the supernatant. Note that the sedimentation of the (+ I) protein required the presence of both spectrin and actin. The positions of molecular weight standards, in kilodaltons, are indicated.
in the pellet when both spectrin and actin were present, in contrast to the isoform that lacked this sequence, which remained in the supernatant. The ability of the (+) motif I isofrom to sediment was dependent on the presence of both spectrin and actin, indicating that the binding was specific for the formation of the ternary complex.

Correas et al. showed that the 10-Kd peptide generated by mild chymotryptic digestion of protein 4.1 formed a ternary complex with spectrin and F-actin with a molar efficiency similar to that of intact protein 4.1. To confirm that the amino acid sequence encoded by motif I was necessary for the association with spectrin and actin in the complex, the function of recombinant 10-Kd peptides that contained or lacked the motif I-encoded sequence was assayed. The 10-Kd peptide that contained the motif I sequence sedimented with spectrin and actin, whereas the peptide that lacked this sequence was found only in the supernatant (Fig 4). Essentially no peptide sedimented alone or with only spectrin or actin (data not shown).

Competition assays were performed to determine whether the interaction of the recombinant (+) motif I isoforms with spectrin and actin were comparable to that of

native erythrocyte protein 4.1. Binding studies were performed as described above, except that an excess of protein 4.1 purified from human erythrocytes was included to compete with the recombinant protein or peptide for binding sites in the ternary complex. The displacement of the [35S]labeled recombinant total protein 4.1 isoform was incomplete, even with a high (>20-fold) molar excess of native protein 4.1 (data not shown), a result that we speculate may be due to the fact that protein 4.1 self-associates, leading to the association of some of the labeled protein molecules with native protein 4.1 molecules that are directly complexed with the spectrin and actin. On the other hand, native protein 4.1 effectively competed with the recombinant (+) motif I 10-Kd peptide, with more than 90% of the peptide displaced by a fivefold molar excess of native protein 4.1 (Fig 5). Half of the peptide is displaced by 0.1 to 0.25 molar equivalents of intact native protein 4.1.

**DISCUSSION**

The diverse isoforms of protein 4.1 arise by alternative splicing of the transcript of a single gene. There are at least 10 motifs that can be selectively included or excluded from the mRNA transcript, in various combinations, to produce these isoforms. This report presents the in vitro demonstration of the function of the first of these motifs, motif I. Motif I is clearly expressed in mature erythroid tissue and may be expressed in other tissues as well. Its expression is inducible during erythroid maturation, suggesting that tissue-specific mRNA processing is capable of producing a protein specifically adapted to its role in the circulating erythrocyte. Our results show that the presence of the 21 amino acid sequence encoded by motif I confers...
the ability to form a ternary complex with spectrin and F-actin on both the full-length protein 4.1 and the 10-Kd spectrin/actin-binding fragment. The association of the recombinant (+) motif I 10-Kd peptide with spectrin and actin in the ternary complex is specific, because native protein 4.1 competitively displaces the peptide from the complex. In fact, the native protein appears to join in the ternary complex somewhat more efficiently than the peptide (Fig 5), suggesting that sequences outside the 10-Kd peptide, although not required for the formation of the complex, may participate in stabilizing the interaction of protein 4.1 with spectrin and actin.

The fact that the spectrin-actin binding domain of protein 4.1 is critical to the maintenance of normal red blood cell shape and membrane stability in vivo is derived from the finding that the domain is absent in protein 4.1 from individuals with hereditary elliptocytosis (HE). HE is an inherited hemolytic anemia characterized by elliptical red blood cell shape and increased membrane fragility. The deletion of this region of protein 4.1 has been found as the sole identifiable defect in cases of HE in both human^4 and the dog.42

In the patients with HE, a shortened form of protein 4.1 (molecular weight of 65/68 Kd) was produced. Two exons encoding 21 and 59 amino acids (lys<sup>407</sup>-gly<sup>465</sup>) were deleted, which includes the chymotryptic peptide that contains the spectrin-actin binding activity (lys<sup>400</sup>-phe<sup>472</sup>). In the case of canine HE, there was a deletion of 63 nucleotides that precisely correspond to the motif I exon. These findings are clearly explained by our data showing that recombinant protein 4.1 and 10-Kd peptide that lack motif I fail to form a ternary complex with spectrin and actin. The ability of protein 4.1 to promote the interaction of spectrin and actin and to anchor the resulting complex to the lipid bilayer is critically important for the normal function of erythrocytes. The formation of the ternary complex by the (+) motif I isoforms, but not by the (−) motif I isoforms of protein 4.1 and the 10-Kd fragment, shows that motif I is necessary for formation of the normal erythrocyte membrane skeleton.

NOTE

While this manuscript was in revision, other investigators reported findings that corroborate the role of the motif I sequence in forming the ternary complex.43

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