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# Isoform Cloning, Actin Binding, and Chromosomal Localization of Human Erythroid Dematin, a Member of the Villin Superfamily*

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**Dematin is an actin-bundling protein of the erythroid membrane skeleton and is abundantly expressed in human brain, heart, skeletal muscle, kidney, and lung. The 48-kDa subunit of dematin contains a headpiece domain which was originally identified in villin, an actin-binding protein of the brush-border cytoskeleton. The headpiece domain of villin is essential for its morphogenic function *in vivo*. Here we report the primary structure of 52-kDa subunit of dematin which differs from the 48-kDa subunit by a 22-amino-acid insertion within its headpiece domain. A unique feature of the insertion sequence of the 52-kDa subunit is its homology to erythrocyte protein 4.2. The insertion sequence also includes a cysteine residue which may explain the formation of sulfhydryl-linked trimers of dematin. Actin binding measurements using recombinant fusion proteins revealed that each monomer of dematin contains two F-actin binding sites: one in the headpiece domain and the other in the undefined N-terminal domain. Although the actin bundling activity of intact dematin was abolished by phosphorylation, no effect of phosphorylation was observed on the actin binding activity of fusion proteins. Using somatic cell hybrid panels and fluorescence *in situ* hybridization, the dematin gene was localized on the short arm of chromosome 8. The dematin locus, 8p21.1, is distal to the known locus of human erythroid ankyrin (8p11.2) and may contribute to the etiology of hemolytic anemia in a subset of patients with severe hereditary spherocytosis.**

Dematin is an actin-bundling protein that was originally identified as a component of the human erythroid membrane skeleton (1–3). Purified dematin consists of two polypeptides of apparent molecular mass of 48 and 52 kDa, respectively (1–3). Recently we reported the primary structure of the 48 kDa subunit of dematin that contains a headpiece domain which was first defined in villin, an actin-binding and -bundling protein (4). The headpiece domain of villin is essential for its actin bundling activity and is required for villin's actin modulating function in the microvillar cytoskeleton (5). Dematin differs from villin in two important respects. 1) In contrast to villin's restricted expression in absorptive epithelia, dematin is expressed widely in many tissues of distinct origin. 2) Unlike villin, the actin bundling activity of dematin is regulated by phosphorylation of dematin by cAMP-dependent protein kinase (2). Based on these distinctive features, it was suggested that dematin may substitute for villin in villin-negative tissues to modulate the organization of filamentous actin in a phosphorylation-dependent manner (4).

In solution, dematin exists as a trimer, and electron microscopy has shown that purified dematin exhibits a trilobed structure (1–3). Each trimer of dematin appears to contain two polypeptides of 48 kDa and one polypeptide of 52 kDa, as estimated by the elution of Coomassie Blue dye from bands after gel electrophoresis (3). Although the two subunits appear to be structurally related, the origin of the 52-kDa subunit remained unknown (3, 4). The recent cloning of the 48-kDa subunit showed that there is only a single cysteine residue in its amino acid sequence (4). This observation implied that there must be an additional cysteine residue in the 52-kDa subunit to account for the formation of sulfhydryl-linked trimers containing two subunits of 48 kDa and one subunit of 52 kDa.

To resolve these issues, we have determined the primary structure of the 52-kDa subunit of dematin. Here we report the amino acid sequence of the 52-kDa subunit which was deduced from the cDNA clones of dematin transcripts present in human reticulocytes. We also show that each subunit of dematin contains two actin binding sites: one in the headpiece domain and the other in an N-terminal undefined domain. Phosphorylation assays demonstrate that the actin binding activity of these domains is not regulated by phosphorylation. Finally, the fluorescence *in situ* hybridization data show that the dematin gene is located distal to the ankyrin locus on the short arm of chromosome 8.

## EXPERIMENTAL PROCEDURES

Glutathione-Sepharose, TA Cloning™ vector, and cAMP-dependent protein kinase were purchased from Pharmacia, Invitrogen, and Sigma, respectively.

*Construction of Recombinant Fusion Proteins*—Constructs representing defined domains of dematin and villin were amplified from the corresponding cDNA templates using the polymerase chain reaction (PCR).[1] The PCR amplified products were subcloned into pGEX-2T plasmid, and fusion proteins were expressed in the *Escherichia coli* strains of DH5α and HB101. Fusion proteins containing glutathione *S*-transferase (GST) were purified by affinity chromatography as described (6). Proteolysis of fusion proteins was reduced by adding leupeptin and phenylmethylsulfonyl fluoride in the solubilization and washing buffers.

The following primers were used to produce the cDNA constructs. 1) Villin headpiece, Ala-753 to Phe-826, sense: 5′GCTAACAGCAACCTC; antisense: 5′TCAAAATAGTCC. 2) Dematin headpiece domain with and

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U28389.*

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[1] The abbreviations used are: PCR, polymerase chain reaction; GST, glutathione *S*-transferase; DAPI, 4,6-diamidino-2-phenylindole; PEST, proline-glutamic acid-serine-threonine-rich regions.

without the insert sequence, Ser-309 to Phe-383, sense: 5′TCAGGGAGTGAGACTGGAAGCCCA; antisense: 5′GAAGAGAGAGGCCTTCTTCTTGA. 3) The truncated headpiece domain of dematin, Pro-362 to Phe-383, sense: 5′CCTGAAGAGTTTGGC; antisense, 5′GAAGAGAGAGGCCTT. 4) N-terminal undefined domain of dematin, Met-1 to Pro-308, sense: 5′ATGGAACGGCTGCAG; antisense: 5′TGGGCTGAACTCCGT. 5) The truncated N-terminal domain, Ser-103 to Pro-308, sense: 5′AGCCGGTCGCCTGGA; antisense: 5′TGGGCTGAACTCCGT. Thirty five cycles of PCR amplification were carried out 55 °C annealing for 1.0 min, 72 °C elongation for 1.0 min, and 94 °C denaturation for 1.0 min.

*Actin Binding Assay*—Actin was isolated from rabbit skeletal muscle as described (7) and further purified by gel filtration on a Sephacryl 300 column. The actin binding assay mixture contained 240 μg/ml actin and an appropriate amount of the GST fusion protein in the following binding buffer: 10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 1.0 mM dithiothreitol, 10 mM KCl, 75.0 mM NaCl. After incubation for 2.0 h at room temperature, actin was sedimented by centrifugation at 35,000 rpm in a Type 42.2 Ti rotor at 4 °C. The supernatant was carefully separated from the pellet, and both fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The amount of proteins bound to actin filaments was quantified by either densitometry or the elution of Coomassie Blue by pyridine (8).

*Other Procedures*—A control peptide was synthesized containing the following sequence: biotin-T-S-P-P-P-S-P-E-V-W. Peptide, biotin-N-E-L-K-K-K-A-S-L-F, corresponds to the C terminus of the dematin headpiece domain. The sequence includes serine 381 which is a consensus site for phosphorylation by cAMP-dependent protein kinase. The two-dimensional peptide mapping was carried out as described (9). Dematin was purified after treating intact red cells with a membrane-permeable protease inhibitor (MDL28170) as well as a protease inhibitor set (Boehringer Mannheim) which was included during subsequent steps of purification. These modifications in the original purification protocol (1, 2) improved the stoichiometry of dematin to 69.9% (48-kDa subunit) and 30.1% (52-kDa subunit).

*Phosphorylation Assay*—Phosphorylation of GST fusion proteins by the catalytic subunit of cAMP-dependent protein kinase was carried out as described (2, 3). Phosphorylation of biotinylated peptides was measured after coupling these peptides to streptavidin-agarose beads. To determine the effect of phosphorylation on the actin binding activity of fusion proteins, purified proteins were phosphorylated by the catalytic subunit of cAMP kinase, and kinase was inactivated at 68 °C as described before (2).

*Chromosome Mapping Using Somatic Cell Hybrids*—Two panels of somatic cell hybrids were used to localize dematin gene on human chromosome. The first panel (A) contains nine human hamster hybrid cell lines containing varying complements of human chromosomes as described previously (10). The second panel is NIGMS monochromosomal somatic cell hybrid panel 2 (11) which was screened as described (12). Selected individual hybrid DNAs were amplified by polymerase chain reaction to confirm assignments based on the pooled DNAs. Twenty-eight to thirty cycles of PCR amplification were carried out in an MJ Research PTC-100 thermal cycler set for 60 °C annealing for 45 s; 72 °C elongation for 30 s; and 94 °C denaturation for 30 s. PCR products were visualized on 1% agarose gels stained with ethidium bromide.

*Isolation of P1 Genomic Clones*—The following primers were used to amplify segments of the dematin gene from human genomic DNA. 1) Sense: OL1, 5′ACAGGAGGAAGAAAGGGAGAG; antisense: OL13, 5′AAACGAGGCAAGTCATCCA. This pair of primers amplified a 120-bp fragment from the 5′-untranslated region of dematin cDNA. 2) Sense: OL4, 5′ATGTCCCCTGAAGAGTTTGGC; antisense: OL6, AACTCTGTGTGCCAGAGCCCA. This pair of primers amplified a 332-bp fragment from the 3′-untranslated region of dematin cDNA. Using a PCR-based screen, the primer pair 4/6 was used to isolate three P1 clones from the DuPont Merck human foreskin fibroblast P1 library (DMPC-HFF1) by Genome Systems, Inc., St. Louis, MO.

*Fluorescence in Situ Hybridization*—Human metaphase chromosomes were prepared from phytohemagglutinin-stimulated lymphocytes of a normal male by standard techniques. Purified DNA from the P1 clone 1977 was labeled with biotin-16-dUTP by nick translation. Hybridization and detection were performed with 150 ng of labeled probe as described previously (13). Hybridization signals were detected with fluorescein-labeled avidin (Vector Laboratories, 7.0 μg/ml). Chromosomes were counterstained with propidium iodide and DAPI. Fluorescein and propidium iodide were visualized through a fluorescein isothiocyanate/Texas red dual band pass filter set (Omega Optical), and DAPI banding was viewed through a single band pass filter set (Zeiss). Photomicrographs were taken with Ektar 1000 color film (Kodak) or ektachrome 400 color slide film (Kodak).

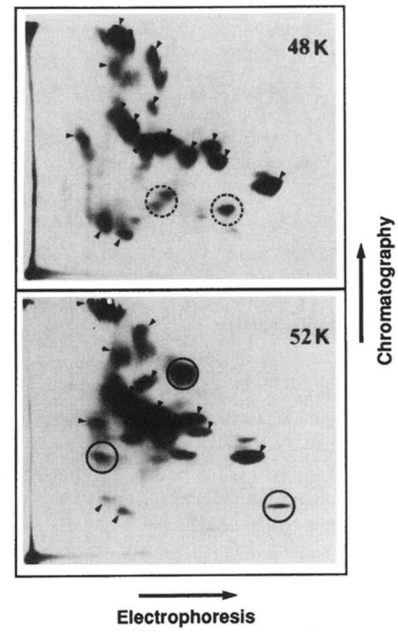
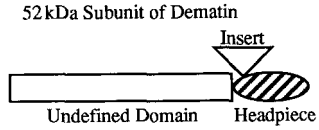



FIG. 1. **Two-dimensional $^{125}$I-tryptic peptide maps.** The 48-kDa and 52-kDa subunits of dematin were separated by electrophoresis of dematin purified from human erythrocyte membranes. The Coomassie-stained polypeptides were excised from the gel, radioiodinated, and digested as described before (9). The spots shared between the 48-kDa and 52-kDa subunits have been identified by *arrowheads*, and *circles* highlight the position of unique spots.

## RESULTS

*Isolation of Human Reticulocyte cDNA Encoding the 52-kDa Subunit*—Despite many similarities between the 48-kDa and 52-kDa subunits of dematin (3), the 52-kDa subunit appears to have subtle structural differences from the 48-kDa subunit based on the following observations. First, the electrophoretic mobility of the 52-kDa subunit changes depending upon the presence and absence of reducing agents, whereas no such mobility shift is observed with the 48-kDa subunit of dematin (3). Secondly, the two-dimensional peptide mapping analysis revealed that although the two subunits of dematin shared many peptides, spots unique to both subunits were also detected (Fig. 1). The respective positions of these unique spots did not change even after an extensive *in vitro* dephosphorylation of purified dematin (not shown). These results indicated that the primary structure of the 52-kDa subunit may be similar to but not identical with that of the 48-kDa subunit of dematin. Based on this assumption, a PCR strategy was designed to amplify short segments of the 48-kDa subunit from total RNA isolated from human reticulocytes. Primers flanking the headpiece domain of the 48-kDa subunit produced a cDNA product that appeared as a doublet on agarose gels (not shown). Subsequent subcloning and sequencing of the cDNA doublet revealed that two distinct cDNA fragments were present in the band. One cDNA encoded the regular headpiece domain of the 48-kDa subunit (4), whereas the second cDNA fragment contained a novel headpiece domain with an additional 66-base pair sequence (Fig. 2). This insert sequence encodes an extra 22 amino acids in-frame, including a cysteine residue, in the headpiece sequence of the 48-kDa subunit (Fig. 2).

We have previously reported that the molecular mass of the 48- and 52-kDa subunits, as measured by the laser desorption mass spectrometry, corresponds to 43,265 Da and 46,175 Da, respectively (4). This difference of 2.91 kDa is in agreement with the 2.2-kDa difference calculated from the insert sequence




B Insert Sequence of 52kDa Subunit of Dematin

1408 AACGGAGAGGGCCAGAGGGGGAGGATG
320 N G E G Q R G R M

1435 GACCGGGGGAACTCCCTGCCCTGTGTG
330 D R G N S L P © V

1462 CTGGAGCAGAAG
340 L E Q K

C Sequence Comparison with Protein 4.2

317 GLQNGEGQRGR (Human 52kDa)
312 GLQNGEGQRGR (Human Protein 4.2)
312 GLQNGEGQRGH (Mouse Protein 4.2)

1399 GGCCTGCAGAACGGAGAG (Human 52kDa)
1121 GGACTTCAGAACGGAGAA (Human Protein 4.2)
1096 GGACTCCAGAATGGAGAA (Mouse Protein 4.2)

1417 GGCCAGAGGGGGAGG (Human 52kDa)
1139 GGCCAGAGAGGCAGA (Human Protein 4.2)
1115 GGACAGAGGGGCCAT (Mouse Protein 4.2)

FIG. 2. **Cloning and sequence alignment of the 52-kDa subunit.** *A*, domain organization of the 52-kDa subunit of dematin. The primary structure of the 52-kDa subunit is identical with that of the 48-kDa subunit except for the insert sequence within the headpiece domain. *B*, the primary structure of the insert sequence encodes 22 amino acids. *C*, a comparison of the insert sequence with protein 4.2. Note that the first three amino acids (GLQ) are contributed from the headpiece domain which is common between the 48-kDa and 52-kDa subunits. The remaining eight amino acids are derived from the insert sequence of the 52-kDa subunit.

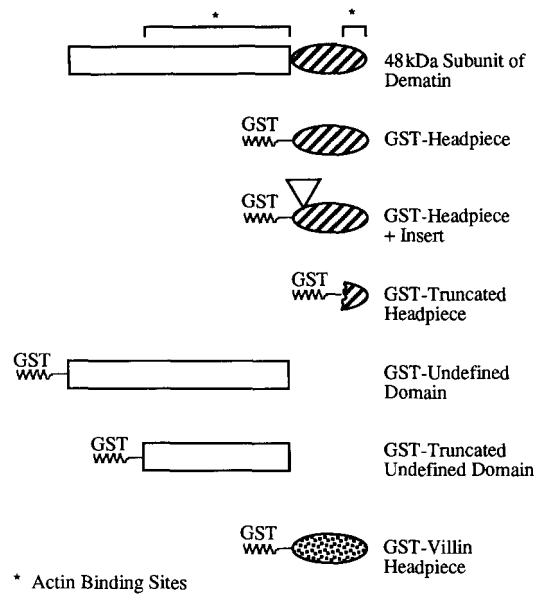



FIG. 3. **Expression of recombinant fusion proteins.** Glutathione *S*-transferase (GST) fusion proteins containing defined domains of dematin, and the headpiece domain of villin, were expressed in bacteria as described under "Experimental Procedures."

of the 52-kDa subunit (Fig. 2). Using insert-specific primers, cDNA clones encoding the full-length 52-kDa subunit were isolated from human reticulocyte mRNA. The primary structure of the 52-kDa subunit is identical with that of the 48-kDa subunit except for the presence of a 22-amino-acid sequence inserted in the headpiece domain of the 52-kDa subunit (Fig. 2). The 52-kDa subunit cDNA clones contained three additional nucleotide changes. The codon for threonine 304 was changed from ACG to ACA without changing the amino acid. The codon for valine 325 (GTG) was changed to methionine (ATG) and serine 358 (TCT) to phenylalanine (TTT). The functional significance of these amino acid substitutions is not currently known. Alternatively, these changes may reflect polymorphisms in the 52-kDa subunit. The observed changes were confirmed by four independent PCR amplifications, and the original codons of the 48-kDa subunit, as reported previously (4), were confirmed.

*The 52-kDa Subunit of Dematin Shows Homology to Erythrocyte Protein 4.2*—Alignment of the 22 amino acids of insert sequence with the available data bank showed that the N-terminal eight amino acids were identical with a motif found in human erythrocyte protein 4.2 (Fig. 2). Additional alignment analysis of the 52-kDa subunit containing the insert sequence revealed homology with protein 4.2 that extended further and included three additional amino acids derived from the headpiece domain just preceding the insert sequence (Fig. 2). Therefore, 11 amino acids of the 52-Da subunit of dematin are identical with a motif present in protein 4.2 (Fig. 2). This 11-amino-acid motif is also conserved in murine protein 4.2 except that a histidine residue has replaced an arginine of the human protein 4.2 as shown in Fig. 2. The functional significance of this shared motif in dematin and protein 4.2 is not yet known.

*Each Monomer of Dematin Subunits Contains Two Actin Binding Sites*—In order to determine the number and location of actin binding sites in dematin, segments of dematin cDNA were expressed in bacteria as glutathione *S*-transferase (GST) fusion proteins (Fig. 3). As shown in Fig. 4 and Table I, the headpiece domain of both 48-kDa and 52-kDa subunits sedimented with actin filaments. The presence of a 22-amino-acid insert sequence did not affect the actin binding activity of the headpiece domain (Fig. 4). To further map the actin binding site within the headpiece domain, a GST fusion protein was made containing the C-terminal 22 amino acids of the headpiece domain (Fig. 4). This fusion protein containing a truncated headpiece domain also sedimented with actin filaments showing that the actin binding site is located within the C-terminal 22 amino acids of the headpiece domain. To further narrow the actin binding site, a synthetic peptide containing the last 10 amino acids of the headpiece domain was produced. This biotin-conjugated peptide, N-L-E-L-K-K-K-A-S-L-F, did not sediment with actin filaments (not shown) indicating that although the consensus sequence for the actin binding site is located within the last 10 amino acids of the headpiece domain (see "Discussion"), the *in vitro* actin binding activity of the headpiece domain requires participation of at least 22 amino acids (Fig. 3).

The primary structure of dematin contains an N-terminal undefined domain which does not show significant sequence homology to any protein in the GenBank™ data base (4). In this respect, dematin differs from villin which has an actin-binding N-terminal domain that is homologous to gelsolin, severin, and fragmin (14, 15). The N-terminal undefined domain of dematin contains a PEST sequence and a highly negatively charged motif of 10 glutamic and aspartic acid residues (4). In order to determine whether this N-terminal domain of dematin contains a second actin binding site, the respective fusion proteins were assayed for actin binding activity. Because of the presence of the PEST sequence, the GST fusion protein containing a full length N-terminal undefined domain was proteolyzed during purification from bacterial lysates (not shown). Nevertheless, this proteolyzed fusion protein containing 25-kDa fragments sedimented with actin filaments (Table I). To further confirm this binding, a GST fusion protein was made which lacked the N-terminal PEST sequence (Fig. 3).

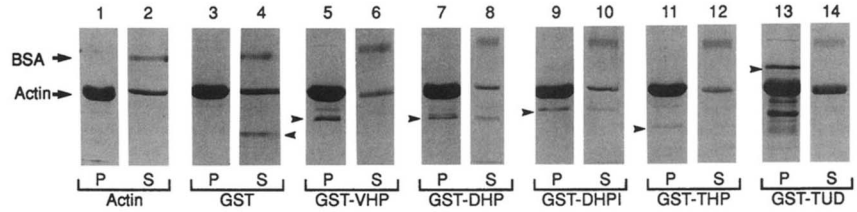



FIG. 4. **Actin binding activity of recombinant fusion proteins.** Specified domains of dematin and villin were expressed in bacteria as glutathione *S*-transferase fusion proteins. The location of respective constructs is described in Fig. 3. Actin binding activity of purified fusion proteins was measured by a sedimentation assay (see "Experimental Procedures"). Bovine serum albumin (*BSA*) was added to reduce nonspecific binding. Coomassie Blue-stained SDS-PAGE pellet (*P*) and supernatant (*S*). The abbreviations are: *GST-VHP*, villin headpiece; *GST-DHP*, dematin headpiece; *GST-DHPI*, dematin headpiece with insert; *GST-THP*, dematin truncated headpiece; *GST-TUD* dematin truncated undefined domain.

TABLE I
*Actin binding and phosphorylation of recombinant fusion proteins*

Purified GST fusion proteins were dialyzed against the binding buffer (see "Experimental Procedures"), and F-actin binding activity of fusion proteins was measured using a sedimentation assay. Purified GST fusion proteins were subjected to phosphorylation *in vitro* by the catalytic subunit of cyclic AMP-dependent protein kinase (see "Experimental Procedures"). Actin binding activity of bacterially produced fusion proteins was quantified by scanning Coomassie-stained polyacrylamide gels. To quantify actin binding activity of phosphorylated fusion proteins, radiolabeled bands were excised from the gels, and radioactivity was measured in a scintillation counter. The experimentally determined value of the pellet was subtracted from the combined total value of supernatant and pellet. The percent binding shown for each supernatant fraction represents a calculated value for that particular construct. Note: due to extensive proteolysis, it was not feasible to produce intact fusion proteins containing either the full-length dematin or truncated undefined domain containing the PEST sequence (residues 90 to 103).

| Glutathione *S*-transferase (GST) fusion proteins | Sedimentation with F-actin | | | |
|---|---|---|---|---|
| | Dephosphorylated (Coomassie Blue) | | Phosphorylated | |
| | Supernatant | Pellet | Supernatant | Pellet |
| | % | | *cpm* | |
| GST | 91.5 | 8.5 | | |
| GST-villin (headpiece) | 1.0 | 99.0 | | |
| GST-dematin (headpiece) | 22.5 | 77.5 | 22.5 | 77.5 |
| GST-dematin (headpiece + insert) | 31.3 | 68.7 | 24.5 | 75.5 |
| GST-dematin (truncated headpiece) | 11.6 | 88.4 | 21.9 | 78.1 |
| GST-dematin (undefined domain) | 9.6 | 90.4 | 16.5 | 83.5 |
| GST-dematin (truncated undefined domain) | 19.6 | 80.4 | 18.5 | 81.5 |

This fusion protein encoding a truncated undefined domain did not undergo proteolysis and also sedimented with actin filaments (Table I). The fusion protein containing only the PEST sequence was extensively proteolyzed and therefore could not be tested for its actin binding activity (not shown). In summary, the results of actin binding measurements show that each subunit of dematin contains at least two actin binding sites: one in the headpiece domain and the other in the N-terminal undefined domain.

*Phosphorylation of Serine 381 by cAMP-dependent Protein Kinase*—The headpiece domain of dematin contains a consensus phosphorylation site for protein kinase A at serine 381 (4). In contrast, the villin headpiece domain contains an alanine residue at the corresponding position (13, 14). Because the actin bundling activity of dematin, but not of villin, is regulated by phosphorylation (2, 3), it was suspected that the phosphorylation of serine 381 may regulate this effect. To test this hypothesis, we first examined whether the headpiece domain of dematin was phosphorylated by protein kinase A *in vitro*. The glutathione *S*-transferase fusion proteins containing headpiece domains of the 48-kDa and 52-kDa subunits were excellent substrates of protein kinase A, whereas no phosphorylation was observed with the headpiece domain of villin (Table I). To further confirm that the site of phosphorylation in the headpiece domain was in fact serine 381, a synthetic peptide was produced that contained the C-terminal 10 amino acids of the headpiece domain of dematin. This synthetic peptide was an excellent substrate of cAMP-dependent protein kinase confirming that serine 381 is indeed the site of phosphorylation (not shown). We then examined whether the actin binding activity of the dematin headpiece domain was regulated by phosphorylation. Actin binding by fusion proteins containing headpiece domains of dematin remained unchanged upon their phosphorylation by protein kinase A (Fig. 5, Table I).

We also examined the possibility that the inhibitory effect of phosphorylation on dematin's actin bundling activity may be mediated via the phosphorylation of an N-terminal undefined domain (Fig. 2). The fusion protein containing the truncated undefined domain of dematin was also phosphorylated *in vitro* by protein kinase A, but again no effect of phosphorylation was observed on its actin binding activity (Fig. 5, Table I). The above results show that although the actin bundling activity of dematin is completely abolished by phosphorylation (2, 3), the actin binding activities of respective domains are not altered by phosphorylation. This result is consistent with our previous observation that the phosphorylated dematin remains associated with actin filaments even though it cannot hold filaments in a bundled conformation (2, 3).

*Dematin Gene Is Located on Human Chromosome 8*—The chromosomal location of the dematin gene was determined using somatic cell hybrid panels and dematin specific primers. The primer pairs OL1/OL13 and OL4/OL6 were combined and used to detect the dematin gene sequences in two somatic cell hybrid panels segregating human chromosomes in a rodent background. These pooled primers did not amplify the dematin gene in control rodent DNAs but segregated with human chromosome 8 in hybrids and were discordant with the segregation of all other human chromosomes (Table II). This assignment was confirmed by positive amplification of the dematin gene in the monochromosomal hybrid GM/NA10156B containing only human chromosome 8 (not shown). These results indicate that the dematin gene is located on human chromosome 8.

Regional localization of the dematin gene on chromosome 8 was performed by fluorescence *in situ* hybridization with a genomic P1 clone of dematin. The primer pair OL4/OL6 was used to isolate three P1 genomic clones specific for human

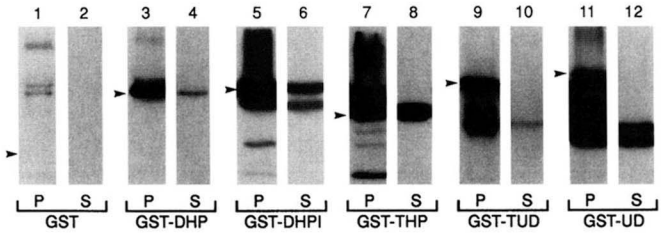



FIG. 5. **Effect of phosphorylation on the actin binding activity of fusion proteins.** Purified fusion proteins were phosphorylated by cAMP-dependent protein kinase (see "Experimental Procedures"). The details of actin binding and abbreviations of fusion proteins are mentioned in Fig. 4. The autoradiograph shows the pellet (*P*) and supernatant (*S*) of the actin binding experiments. Note that the *in vitro* phosphorylation had no effect on the binding of fusion proteins to actin filaments. Villin headpiece is not shown because it was not phosphorylated under these conditions. The undefined domain of dematin (*GST-UD*) is included to highlight the extreme sensitivity of this construct to proteolysis.

TABLE II
*Segregation of dematin gene PCR products with human chromosomes in DNAs from somatic cell hybrids*

| Chromosome[a] | Hybridization pattern[b] | | | | Discordant fraction[c] |
|---|---|---|---|---|---|
| | +/+ | −/− | +/− | −/+ | |
| 1 | 2 | 6 | 7 | 1 | 0.50 |
| 2 | 3 | 5 | 5 | 1 | 0.43 |
| 3 | 3 | 4 | 5 | 3 | 0.53 |
| 4 | 4 | 6 | 4 | 1 | 0.33 |
| 5 | 4 | 4 | 5 | 3 | 0.50 |
| 6 | 3 | 3 | 6 | 3 | 0.60 |
| 7 | 2 | 3 | 7 | 4 | 0.69 |
| 8 | 9 | 7 | 0 | 0 | 0.00 |
| 9 | 3 | 6 | 6 | 1 | 0.44 |
| 10 | 5 | 4 | 3 | 2 | 0.36 |
| 11 | 1 | 3 | 7 | 4 | 0.73 |
| 12 | 3 | 3 | 6 | 3 | 0.60 |
| 13 | 2 | 3 | 7 | 4 | 0.69 |
| 14 | 4 | 4 | 4 | 3 | 0.47 |
| 15 | 1 | 4 | 8 | 3 | 0.69 |
| 16 | 5 | 4 | 4 | 3 | 0.44 |
| 17 | 1 | 3 | 8 | 4 | 0.75 |
| 18 | 3 | 4 | 6 | 4 | 0.59 |
| 19 and 19der[d] | 6 | 3 | 3 | 4 | 0.44 |
| 20 | 7 | 4 | 2 | 3 | 0.31 |
| 21 | 5 | 5 | 4 | 2 | 0.38 |
| 22 | 4 | 5 | 5 | 2 | 0.44 |
| X | 2 | 6 | 6 | 1 | 0.47 |
| Y | 1 | 6 | 8 | 1 | 0.56 |

[a] Data are combined from two hybrid panels described under "Experimental Procedures."

[b] Number of hybrids with indicated pattern of PCR products and chromosomes. Column designations: +/+, PCR products and chromosome both present; −/−, PCR products and chromosome both absent; +/−, PCR products present but chromosome absent; −/+, PCR products absent but chromosome present.

[c] Hybrids with a rearranged chromosome or in which the chromosome was present in fewer than 15% of cells were excluded for calculation of discordant fractions.

[d] This includes nine hybrids containing the der 19 translocation chromosome 19pter-q13::Xq24-qter.

dematin from the DuPont Merck P1 library (see "Experimental Procedures"). The identity of these clones was established by Southern blot analysis (not shown). The DNA from one of the three P1 clones, designated P1-1977, was biotinylated and hybridized to human chromosomes. Fifteen metaphases with single or double chromatid hybridizations were examined using the P1-1977 genomic probe. All revealed hybridization on the short arm of chromosome 8. The dematin gene was localized to 8p21.1 by simultaneous visualization of the hybridization signals on DAPI-banded chromosomes and by fractional length measurements (Fig. 6).

## DISCUSSION

In this manuscript, we describe the complete primary structure of the 52-kDa subunit of dematin, an actin-bundling phosphoprotein. The amino acid sequence contains an N-terminal undefined domain and a C-terminal headpiece domain, an organization similar to that of the 48-kDa dematin subunit (Fig. 2, Table III). However, the headpiece domain of the 52-kDa subunit contains an additional sequence of 22 amino acids suggesting that this isoform arises from the inclusion of an alternatively spliced exon that is not present in the 48-kDa isoform. This inserted sequence starts after glutamine 319 of the headpiece domain of the 52-kDa subunit (Fig. 2). The elucidation of the primary structure of the 52-kDa subunit may explain the formation of trimeric dematin in solution. Each trimer of dematin appears to consist of two polypeptides of 48 kDa and one polypeptide of 52 kDa (3). Because of the presence of only one cysteine residue in the 48-kDa subunits (4), it was postulated that there must be an additional cysteine residue in the 52-kDa subunit to allow formation of disulfide-linked trimers. As proposed in Fig. 7, the second cysteine in the insert sequence of the 52-kDa subunit should allow formation of a disulfide-linked trimer between the 48-kDa and 52-kDa subunits. This result is also consistent with our previous observation that the 52-kDa subunit exhibits decreased mobility in the absence of dithiothreitol, a reducing agent, whereas the mobility of the 48-kDa subunit remained the same regardless of whether dithiothreitol was present (3). It is relevant to note that the disulfide-linked proteins are often resistant to reduction *in vivo* (16), and, thus, the presence of sulfhydryl-linked trimers of dematin may be of physiological significance in intact cells.

A unique feature of the insert sequence in the 52-kDa subunit is its homology with erythrocyte protein 4.2 (Fig. 2). The 11 amino acids of the 52-kDa subunit are identical with a motif present in protein 4.2. Of these 11 amino acids, 8 residues are contributed from the insert sequence of 52 kDa, and 3 amino acids are derived from the original headpiece domain (Fig. 2). Although the function of this motif is not yet known, it is highly conserved between human and murine protein 4.2 which are considerably different in their primary structures (17). Interestingly, this conserved motif is not found in transglutaminases which share significant sequence identity with human and murine protein 4.2 (18). These observations raise the possibility that the role of this motif may be specific to the functions of dematin and protein 4.2 and is currently under investigation.

The availability of the primary structures of dematin subunits allowed us to map the location of actin binding sites within respective domains of each subunit (Fig. 3). Each dematin subunit contains two actin binding sites: one in the headpiece domain and the other in the undefined N-terminal domain. The actin binding site in the headpiece domain is located within the C-terminal 22 amino acids (Fig. 3). The presence of an insert sequence of 22 amino acids in the headpiece domain of the 52-kDa subunit has no effect on the actin binding activity of this isoform *in vitro*. The location of an actin binding site in the headpiece domain is consistent with the localization of an actin binding site in the villin headpiece domain (5). An attempt to further reduce the primary sequence of the actin binding site in the dematin headpiece domain was not successful, perhaps because of insufficient folding of the synthetic peptide. A similar observation has been made previously with the headpiece domain of villin (5).

A protein monomer must contain at least two actin binding sites to function as an actin-bundling protein. Alternatively, self-association of monomers containing a single actin binding site can also provide multiple sites for actin binding. Our re-

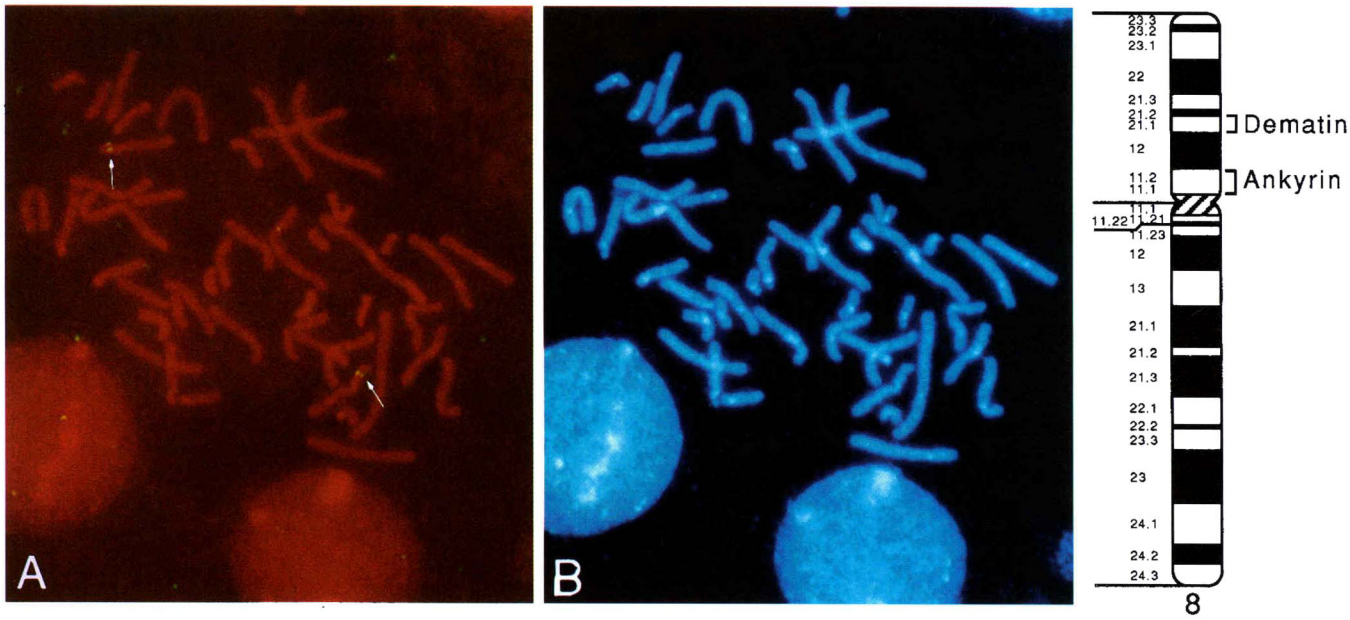



FIG. 6. **Localization of genomic dematin clone 1977 to human chromosome 8p21.1 by fluorescence *in situ* hybridization.** *A*, both chromatids are hybridized on each chromosome 8 (*arrows*). *B*, corresponding DAPI-banded chromsomes. The hybridized chromatids (two for each chromosome) are indicated. Note: the signals were not amplified with anti-avidin so as to reduce nonspecific background hybridization.

TABLE III
*A comparison of the properties of dematin subunits*

| Properties | Dematin | |
|---|---|---|
| | 48-kDa subunit | 52-kDa subunit |
| Amino acids | 383 | 405 |
| Calculated molecular mass[a] | 43,121 | 45,548 |
| SDS-PAGE[b] molecular mass | 48,000 | 52,000 |
| Laser desorption molecular mass | 43,265 | 46,175 |
| Calculated isoelectric point[a] | 9.54 | 9.54 |
| Estimated charge at pH 7.0[a] | 5.83 | 6.79 |
| Number of cysteines | 1 | 2 |
| Actin binding | Yes | Yes |
| Homology with protein 4.2 | No | Yes |

[a] Calculated using the PC-GENE program from Intelligenetics.
[b] PAGE, polyacrylamide gel electrophoresis.

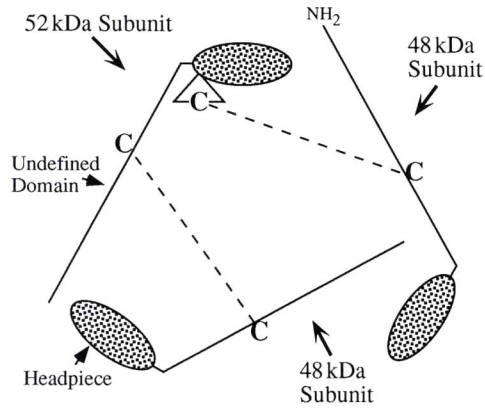



FIG. 7. **A proposed model for the formation of trimeric dematin.** The presence of a second cysteine residue in the insert sequence of the headpiece domain is sufficient to form a disulfide-linked trimer between a 52-kDa subunit and two subunits of 48 kDa. The chemical cross-linking experiments have previously shown that both subunits of dematin are cross-linked via disulfide bonds in dematin trimers (1, 3).

sults show that both subunits of dematin contain a second binding site for F-actin in their N-terminal undefined domains and hence allow a dematin trimer to hold six actin filaments (Fig. 3). Moreover, it appears that the actin binding activity of fusion proteins containing either the headpiece domain or undefined domain was not influenced by the state of their phosphorylation (Table I, Fig. 5). This result is consistent with our previous observation that the phosphorylated dematin remains bound to actin filaments although it cannot bundle actin filaments (2, 3). Since both dephospho- and phosphorylated forms of dematin exist as trimeric particles (1–3), we propose that phosphorylated dematin fails to bundle actin filaments because it cannot hold filaments in correct orientation due to phosphorylation-induced conformational changes. If true, the proposed model may also explain the rapidity with which dematin regains its actin bundling activity upon dephosphorylation (2, 3). The experimental evidence in support of this model will come from elucidation of dematin's three-dimensional structure in both dephosphorylated and phosphorylated forms.

The results of somatic cell hybrids suggest that there is a single copy of the dematin gene on chromosome 8. Further analysis by fluorescence *in situ* hybridization revealed that the dematin gene is located on the short arm of chromosome 8 (8p21.1), which is distal to the SPH2 locus containing the ankyrin gene (8p11.2) (19). Previously, several groups have shown that the locus for hereditary spherocytosis resides on the short arm of human chromosome 8 (20, 21). Lux *et al.* (19) have demonstrated that a single copy of the ankyrin gene is missing in two children with severe hereditary spherocytosis due to a heterozygous deletion (p11-p21.1) of chromosome 8. Since red cell membrane proteins spectrin, actin, protein 4.1, protein 4.2, and band 3 are not located on chromosome 8, it was suggested that the partial deficiency of ankyrin 1 leads to severe hereditary spherocytosis (19). Our finding showing the location of dematin gene on the short arm of chromosome 8 raises the possibility that the abnormalities in the dematin gene may also contribute to the genesis of hereditary spherocytosis perhaps by destabilizing the actin binding end of spectrin tetramers in mutant erythrocytes (22). Alternatively, the mutant dematin gene may functionally manifest in the abnormalities of nonerythroid tissues. Interestingly, a subset of hereditary spherocytosis patients with chromosome 8 deletions suffers from multiple neurological defects (19, 20), and the abundant expression of dematin in human brain may be of physiological consequence in this context.

While our search continues for a physiological basis of dema-

tin's actin bundling activity, it was considered necessary to elucidate the primary structure of its two subunits. This information may help to explain the assembly of dematin subunits into trimers and sheds light on the mechanism by which phosphorylation regulates dematin's actin bundling activity. We have previously suggested that, in mature erythrocytes, dematin may function to link spectrin-actin complexes to the plasma membrane, a function akin to another adaptor protein 4.1 (4, 22). To accomplish its function in mature erythrocytes where actin bundles appear to be absent, the actin binding activity of dematin appears to be sufficient whereas dematin's actin bundling activity may be functionally more relevant during erythropoiesis. Dematin is expressed in early erythroblasts[2] where actin bundles do exist (23). In fact, actin bundling events have been correlated with the process of enucleation in murine erythroblasts (23). Whether dematin plays a role in such actin bundling events during erythoid development is currently under investigation.

Dematin is a member of the villin superfamily by virtue of a common C-terminal headpiece domain (4). It is noteworthy that other members of the villin superfamily such as gelsolin, severin, and fragmin lack villin's headpiece domain and therefore do not bundle actin filaments (24). The presence of a headpiece domain therefore appears to be required for the actin bundling function. To date, three proteins are known to contain the headpiece domain. In villin, the headpiece domain is necessary for the actin bundling activity and is essential for villin's morphogenic function *in vivo* (5). Recently, the Drosophila *quail* gene has been shown to encode a villin-like protein with a headpiece domain (25). *Drosophila* villin-like protein is germline-specific, and its null mutations result in female sterility due to an abnormal transport of cytoplasm from nurse cells into the oocyte (25). The primary molecular defect appears to be the failure of actin bundle assembly in mutant nurse cells, demonstrating that the villin-like protein functions by bundling actin filaments *in vivo* (25). Unlike villin and *Drosophila* villin-like protein, dematin is unique in its widespread tissue distribution and is the only actin-bundling protein with a headpiece domain whose activity is reversibly controlled by phosphorylation (2, 3). A dematin-like protein, RET52, has been suggested to play a role in the regulation of disk membrane assembly and synapse formation within photoreceptors (26). Now that the primary structure of the dematin subunits has been elucidated, it will be possible to co-express a reconstituted trimer in a heterologous expression system in order to assess the biological function of dematin *in vivo*.

[2] A. C. Azim, J. H. M. Knoll, A. H. Beggs, and A. H. Chishti, unpublished data.

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