A Phosphorylation-Induced Conformation Change in Dematin Headpiece

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Summary

Dematin is an actin binding protein from the junctional complex of the erythrocyte cytoskeleton. The protein has two actin binding sites and bundles actin filaments in vitro. This actin bundling activity is reversibly regulated by phosphorylation in the carboxyl terminal “headpiece” domain (DHP). DHP is a typical villin-type headpiece actin binding motif and contains a flexible N-terminal loop and an \(\alpha\)-helical C-terminal subdomain that is phosphorylated at Ser74. The NMR structure of a Ser74-to-Glu mutant (DHPs74e) closely mimics the conformation of phosphorylated DHP. The negative charge at Ser74 does not alter the conformation of the C-terminal subdomain, largely due to alternative RNA splicing (Azim et al., 1995; Kim et al., 1998). The 22 amino acid insertion of dematin differs from the 48 kDa isoform by an insertion of 22 amino acids near the N terminus of the headpiece domain due to alternative RNA splicing (Azim et al., 1995; Kim et al., 1998). The 22 amino acid insertion is largely unfolded in the isolated headpiece domain from the 52 kDa isoform (Frank et al., 2004).

The headpiece domain in dematin is essential for the function of the protein and the correct maintenance of the erythrocyte cytoskeleton. Dematin headpiece knockout mice are viable, but they develop compensated anemia and spherocytosis (Khanna et al., 2002). Without the headpiece domain, the association of spectrin and actin to the plasma membrane is weakened, leading to osmotically fragile erythrocytes with reduced deformability and filterability (Khanna et al., 2002). The headpiece not only provides one actin binding site, but also contains the phosphorylation site that reversibly regulates the actin bundling activity of dematin. In vitro, cyclic AMP-dependent protein kinase specifically phosphorylates dematin at Ser381 (Ser74 in DHP) and abolishes the actin bundling activity (Husain-Chishti et al., 1988, 1989). The inhibition works on dematin in both monomer and trimer forms (Husain-Chishti et al., 1988). The actin bundling activity of dematin is restored after treatment with phosphatase (Husain-Chishti et al., 1988). Surprisingly, phosphorylation does not eliminate the actin binding activity of either the N-terminal core domain or the headpiece domain (Azim et al., 1995; Vardar et al., 2002). This phosphorylation-mediated actin bundling activity of dematin may underlie an important regulation step in the signal transduction cascade that affects cytoskeletal organization and may account for the phenotype of the dematin headpiece knockout mice. Because the mature erythrocyte contains very few actin filaments and lacks actin bundles, this phosphorylation regulation may occur during erythrocyte development, when actin bundles do exist. Despite the unresolved physiological roles, the understanding of the structural basis for this phosphorylation-regulated bundling activity becomes possible with the NMR structure of dematin headpiece.

Headpiece is a highly conserved actin binding domain in a variety of proteins. However, the conservation of the N-terminal subdomain of headpiece is much lower than that of the C-terminal subdomain, largely due to the variable length of the V-loop (Vardar et al., 1999). This region ranges from 2 to 10 residues in the known headpiece sequences. Dematin has a 10 residue V-loop (residues 21–28), the longest in the headpiece family. The V-loop of dematin is disordered in the DHP NMR structure (Frank et al., 2004). Furthermore, \(^{\text{15}}\)N-relaxation experiments provide the structural basis for this phosphorylation-regulated bundling activity becomes possible with the NMR structure of dematin headpiece.

Introduction

Dematin, also named band 4.9, is an actin binding protein abundant in a variety of tissues including human brain, heart, blood, skeletal muscle, kidney, and lung (Kim et al., 1998). Previously known as band 4.9, the protein was originally purified from the junctional complex of the spectrin cytoskeleton that supports the erythrocyte membrane (Siegel and Branton, 1985). In addition to its structural role in erythrocytes, dematin interacts with the Ras-guanine nucleotide exchange factor Ras-GRF2 and modulates mitogen-activated protein kinase pathways (Lutchman et al., 2002). The loss of heterozygosity of the dematin gene is also implicated in prostate cancer (Lutchman et al., 1999). Dematin purified from erythrocytes exists as a trimer, composed of two copies of a 48 kDa polypeptide and one copy of a 52 kDa polypeptide (Azim et al., 1995). Even though dematin is well characterized as an actin bundling protein in vitro, the exact function of the protein in vivo remains unclear.

Dematin consists of a ~300 residue, unique N-terminal core domain and a 76 residue headpiece domain at the extreme C terminus. The N-terminal core domain contains one actin binding site and is not homologous to any known protein sequence. It has several segments with low compositional complexity that are likely to be unfolded in solution. The C-terminal 76 amino acids of dematin belong to the villin-type headpiece family and bind F-actin. The structure of the 48 kDa form of dematin headpiece domain (DHP) was determined by solution NMR (Frank et al., 2004). DHP is composed of a well-folded helical C-terminal subdomain, which is very similar to the same region in the villin headpiece (VHP), and a more mobile N-terminal subdomain, containing mainly loops and turns, including an unstructured variable length loop (V-loop) that varies in length among headpiece sequences (Frank et al., 2004). The 52 kDa isoform of dematin differs from the 48 kDa isoform by an insertion of 22 amino acids near the N terminus of the headpiece domain due to alternative RNA splicing (Azim et al., 1995; Kim et al., 1998). The 22 amino acid insertion is largely unfolded in the isolated headpiece domain from the 52 kDa isoform (Frank et al., 2004).

The headpiece domain in dematin is essential for the function of the protein and the correct maintenance of the erythrocyte cytoskeleton. Dematin headpiece knockout mice are viable, but they develop compensated anemia and spherocytosis (Khanna et al., 2002). Without the headpiece domain, the association of spectrin and actin to the plasma membrane is weakened, leading to osmotically fragile erythrocytes with reduced deformability and filterability (Khanna et al., 2002). The headpiece not only provides one actin binding site, but also contains the phosphorylation site that reversibly regulates the actin bundling activity of dematin. In vitro, cyclic AMP-dependent protein kinase specifically phosphorylates dematin at Ser381 (Ser74 in DHP) and abolishes the actin bundling activity (Husain-Chishti et al., 1988, 1989). The inhibition works on dematin in both monomer and trimer forms (Husain-Chishti et al., 1988). The actin bundling activity of dematin is restored after treatment with phosphatase (Husain-Chishti et al., 1988). Surprisingly, phosphorylation does not eliminate the actin binding activity of either the N-terminal core domain or the headpiece domain (Azim et al., 1995; Vardar et al., 2002). This phosphorylation-mediated actin bundling activity of dematin may underlie an important regulation step in the signal transduction cascade that affects cytoskeletal organization and may account for the phenotype of the dematin headpiece knockout mice. Because the mature erythrocyte contains very few actin filaments and lacks actin bundles, this phosphorylation regulation may occur during erythrocyte development, when actin bundles do exist. Despite the unresolved physiological roles, the understanding of the structural basis for this phosphorylation-regulated bundling activity becomes possible with the NMR structure of dematin headpiece.

Headpiece is a highly conserved actin binding domain in a variety of proteins. However, the conservation of the N-terminal subdomain of headpiece is much lower than that of the C-terminal subdomain, largely due to the variable length of the V-loop (Vardar et al., 1999). This region ranges from 2 to 10 residues in the known headpiece sequences. Dematin has a 10 residue V-loop (residues 21–28), the longest in the headpiece family. The V-loop of dematin is disordered in the DHP NMR structure (Frank et al., 2004). Furthermore, \(^{\text{15}}\)N-relaxation experiments...
indicate a higher than average mobility in the V-loop (Frank et al., 2004). Interestingly, after phosphorylating Ser74, chemical shift changes indicate a conformational change in the V-loop region of DHP (Frank et al., 2004). Here, we report the structure of a DHP mutant that replaces Ser74 with Glu, which closely mimics the conformation of the phosphorylated DHP, to illuminate the atomic details of this conformational change. We also examine the effect of the mutation on the dynamics of the V-loop.

Results

The s74e Mutation Mimics the Conformation Change Induced by Phosphorylation

Production of large amounts of phosphorylated DHP is complicated by low yield and multiple phosphorylations. In order to overcome the yield and homogeneity problems in the phosphorylation reaction, we mutated Ser74 to Glu, mimicking the negative charge of the phosphate group. The conformations of DHP, phosphorylated DHP (DHP-Pi), and DHPs74e are compared via their 15N-HSQC spectra (Figure 1A). In 15N-HSQC spectra, the chemical shift of the individual peaks sensitively reflects the local environment of the corresponding amide bond. While we have not been able to produce sufficient amounts of DHP-Pi for assignment, the close correspondence of the peaks of DHP-Pi and DHPs74e, especially in the more disperse regions of the spectra, indicates that their structures are highly similar, yet different from DHP (Figure 1A). These observations strongly indicate that DHPs74e adopts a very similar conformation to that induced by the phosphorylation of DHP.

Specifically, residues 17–18, 23–29, and 32–35 in the DHP-Pi and DHPs74e spectra have almost identical chemical shift changes that differ from DHP (Figures 1B and 1C). Most changes in DHPs74e chemical shifts are in the same direction as in DHP-Pi, but they are slightly less in magnitude. Residues 19–21 and 71–76 in DHPs74e have chemical shift changes not identified in the DHP-Pi spectrum (Figures 1B and 1C). Among these residues, residue 20 is not observable and residue 21 is extremely weak in the DHPs74e spectrum, which makes it difficult to find the corresponding peaks in the DHP-Pi spectrum. The chemical shift changes of residues 71–76 in DHPs74e are the result of a larger local environment change due to the Ser-to-Glu substitution and are not observed in DHP-Pi. Apparently, Ser-to-Glu mutation has a more pronounced local perturbation than the phosphorylation of Ser. The magnitude of the chemical shift changes decreases, as residues move farther away from the site of mutation. Thr22 is the only one with a chemical shift change in the opposite direction in the DHP-Pi and DHPs74e spectra, but the magnitude of the change is small (≤0.1 ppm). In summary, the phosphorylation of DHP and the Ser74-to-Glu mutation result in an extremely similar conformation change, primarily involving residues 17–35. All peaks, with the exception of Thr22, Pro31, and Pro32, which are not observable, have directly correlated chemical shift changes between the DHPs74e and DHP-Pi 15N-HSQC spectra.

NMR Structure of the Dematin Headpiece s74e Mutant

Like DHP, the DHPs74e mutant gives high-quality NMR spectra (Figure S1; see the Supplemental Data available with this article online). In the 15N-HSQC spectrum, two peaks, Gly10 and Val20, are missing. As in DHP, Gly10 is only observed below 15°C. Val20, a strong peak in the DHP spectrum, is only observed at temperatures above 30°C in DHPs74e. About half of the proton chemical shifts do not change significantly after the Ser-to-Glu mutation. Side chain assignments are essentially complete, except for the side chain atoms in Lys70, in which the γ, δ, and ε protons are degenerate.

The structure of the DHPs74e was calculated by using a combined distance geometry/simulated annealing
approach with the CNS software package (Brunger et al., 1998). Of the 100 initially calculated structures, 35 structures were accepted with no NOE violation greater than 0.5 Å and no dihedral angle violation greater than 5º. The 20 lowest-energy structures were chosen to calculate the average structure (Figures 2A and 2B). The statistics of the 20 reported structures are presented in Table 1. The root mean square (rms) deviation of the backbone heavy atoms and all heavy atoms are 0.52 Å and 0.94 Å, respectively. Omitting the V-loop region, the rms deviation for residues 12–20 and 29–76 is 0.32 Å for backbone heavy atoms and 0.80 Å for all heavy atoms.

As previously reported, the structure of DHP can be divided into two subdomains, the N-terminal subdomain (residues 9–42) and the C-terminal subdomain (residues 43–76). In DHP, the N-terminal subdomain primarily contains bends and turns, with little secondary structure, while the C terminus is tightly folded with three α helices, spanning residues 44–50, 55–60, and 63–73. DHPs74e maintains the same two-subdomain characteristics of DHP; however, a large conformation change is observed in the N-terminal subdomain (Figure 2C). The V-loop in DHPs74e adopts a new fold distinct from any of the 12 DHP structures reported previously.

The C-terminal subdomain of DHPs74e consists of the same three α helices as in DHP, except that the last α helix spans residues 63–72, one residue shorter than the helix in DHP, which extends to residue 73. This perturbation of the C terminus could be a result of the nearby Ser74-to-Glu mutation. The C-terminal subdomains of DHPs74e and DHP fit with each other very well with a backbone rms deviation of 0.98 Å. Most of the residues that make up the hydrophobic core of the C terminus of DHP, including residues Phe47, Phe51, Phe58, Leu69, Ala73, and Leu75, remain buried in DHPs74e. Compared to DHP, residues Arg66 and Lys70 increased their solvent accessibility from 4.1% and 0.7% to 24.3% and 10.7%, respectively. In DHPs74e, the side chain of Arg66 is more solvent accessible and located farther away from Glu39, which precludes the formation of a salt bridge with Glu39. This change could potentially be an explanation for the decrement in actin binding affinity of the phosphorylated DHP (Azim et al., 1995; Varadar et al., 2002). Lys70 is located in close proximity to Glu39 (6.8% solvent accessibility), indicating a possible
buried salt bridge, which is also observed in both DHP and villin headpiece HP67 (Vardar et al., 1999).

A more drastic conformation change is observed in the N-terminal half of the DHPs74e structure. The N-terminal subdomains (residues 9–42) of DHPs74e and DHP fit poorly with each other, with an rms deviation of 2.42 Å. The conformation of the V-loop (residues 20–28) in the N-terminal subdomain of DHPs74e is much better determined than that in DHP, in which the V-loop is almost unstructured due to the lack of constraints. A comparison of the number of NOE distance constraints for each residue indicates a significant increase in interresidue NOE constraints for residues in the V-loop in the DHPs74e spectra compared to DHP (Figure S2). Notably, in DHPs74e, the V-loop moves closer to the C terminus. Seventeen NOEs are identified between residues 19–23 in the V-loop and residues 74–76 in the C-terminal helix. In contrast, no such NOEs are observed in the wild-type DHP spectra. Interestingly, the amide proton in Val20 is not observed at 20°C, but six NOE peaks are observed between its side chains and residues Glu74 and Leu75. The amide proton of Val20 appears to be in intermediate exchange, possibly acting as a hinge for the loop.

Most of the N-terminal hydrophobic core residues in DHP, including Tyr14, Tyr16, Leu19, Pro30, Val33, Leu38, Glu39, His41, and Leu42, remain buried in DHPs74e. In addition, Val20 and Val21 significantly decrease their solvent accessibility from 15.1% and 26.1% to 0.4% and 2.3%, respectively. Leu29 also becomes buried by forming contacts with Pro30, Leu19, and Leu38. Such contacts as well as the new hydrophobic core formed by Leu19, Val20, Val21, Leu75, and H2 of Glu74 may stabilize the V-loop in the new orientation observed in DHPs74e (Figure 2D).

The fact that the Ser74-to-Glu mutation closely mimics the conformation change by phosphorylating DHP indicates that electrostatic interactions are important driving forces. Not surprisingly, in DHPs74e, Glu74 is found to be involved in a potential salt bridge with Lys24 (Figure 2E). The closest carboxyl oxygen in Glu74 is merely 2.4 Å away from the amino proton in Lys24 in the average structure. Among the 20 accepted structures, 17 structures contain at least one oxygen in Glu74 within 5 Å from the ε-amide proton in Lys24. Because the ε-proton in Lys24 is rapidly exchanging with the solvent, direct NOE observation of Lys Hε is not possible. Indeed, if Lys24 and Glu74 do form a head-to-head salt bridge, no NOE should be observed between these two residues, as the closest protons, Hε in Lys24 and Hγ in Glu74, would be more than 5 Å apart. Addition of a 1.8 Å distance constraint between the amino proton in Lys24 and the carboxyl oxygen in Glu74 has essentially no change in the overall energy of the calculated structure, suggesting that such a salt bridge is completely compatible with the current structure. However, including similar constraints between Glu74 and the other positively charged residues, Arg26 or Lys28 in the V-loop, results in multiple distance/angle violations and significant energy increases.

### A Change in the Mobility of the V-Loop

The measurement of the 15N-relaxation rates indicates that the V-loop in DHP has a much higher mobility in solution compared to the C-terminal half of the protein (Figures 3A–3C). This difference is especially striking in the heteronuclear NOE experiment, in which the peak intensity is compared with and without presaturation. Other than the N-terminal 3 residues, the heteronuclear NOE values of residues 21–39 are clearly less than the rest of the protein (Figure 3C). The heteronuclear NOE difference is in good agreement with the rms deviation of the DHP NMR structures, in which the N terminus and the V-loop are much less well defined (Frank et al., 2004). The relaxation data suggest that the unstructured V-loop in DHP is not a result of the low number of constraints, but of a true flexibility in this region.

After the Ser74-to-Glu mutation, the V-loop in DHPs74e has a reduced difference in relaxation rates compared to the rest of the protein (Figures 3D–3F). This change is most pronounced in the heteronuclear...
Compared to the average heteronuclear NOE values (excluding residues 21–29), the following heteronuclear NOE decreases are observed: Thr22, 4%; Asn23, 33%; Lys24, 29%; Gly25, 44%; Arg26, 22%; Thr27, 14%; Lys28, 19%; Leu29, 4%. These changes are consistent with the fact that the DHPs74e has a better-defined V-loop in the NMR structure. However, in general, the V-loop in DHPs74e is still more mobile than the C-terminal subdomain, suggesting that the conformation state of the V-loop is less stable.

In addition to the change in the heteronuclear NOE values, the R2 values for DHPs74e are significantly reduced compared to DHP, while the R1 values are similar for both. The mechanism for the reduction in R2 values is not clear and is currently under investigation. It is possible that the ordering of the V-loop in DHPs74e results in the loss of motions on the microsecond to millisecond timescale that are pathways for R2 relaxation in DHP. The loss of motion could result in the lower R2 relaxation rates observed for DHPs74e than for DHP. However, the effect may also be due to changes in overall tumbling or aggregation effects.

**Discussion**

The villin-type headpiece domain is an actin binding module present in a large number of proteins. So far, about 146 sequences encoding headpiece homologs have been found. Surprisingly, in all reported headpiece-containing proteins, the headpiece is always found at the extreme C terminus. As illustrated in the three determined headpiece structures—villin, dematin, and an actin binding Lim protein homolog (KIAA0943, 1UJS.PDB)—the domain is composed of a highly conserved three-helix motif at the C terminus and a more variable N terminus, consisting mainly of loops and turns (Figure 4A) (Frank et al., 2004; Vardar et al., 1999). The 35 amino acid C-terminal subdomain of chicken and human villin and human advillin headpiece (HP35) fold into their native conformations in isolation, stabilized by three Phe residues (Frank et al., 2002; McKnight et al., 1997; Vermeulen et al., 2004). However, HP35 has a significantly reduced actin binding affinity, suggesting that the N-terminal region is also important for high-affinity actin binding (Vardar et al., 2002; Vermeulen et al., 2004). Vardar et al. (2002) proposed that three structural elements in the headpiece domain are critical for actin binding: (1) a hydrophobic cap formed by Trp64 and Leu63, (2) an alternating charged “crown” below the hydrophobic cap, and (3) a positive patch below the cap and the “crown” (Figure 4B). Residues in the “crown” and the positive patch seem to be able to tolerate certain variation (Rossenu et al., 2003). The N-terminal subdomain is more diverse in both sequence and conformation in the three determined structures (Figure 4B). In dematin headpiece, the N-terminal subdomain also responds to a phosphorylation signal in the C-terminal subdomain, which mediates the actin bundling ability of the full-length protein.

Dematin is the only protein in the headpiece family that contains a Ser residue at position 74, which is normally a Gly or positively charged residues in other headpiece proteins. Although the physiological function remains undetermined, phosphorylation at Ser74 dramatically inhibits the actin bundling activity of dematin in vitro (Husain-Chishti et al., 1988, 1989). In order to understand the structural basis of this change, we mimicked the conformation of DHP-Pi by replacing the negatively charged phosphoserine with a glutamic acid in the DHPs74e mutant, thus allowing the structure determination. For nearly every peak in the DHPs74e HSQC spectrum, there is a corresponding DHP-Pi peak in close proximity, especially in well-dispersed regions. Most of the dispersed peaks of DHPs74e move away...
from the DHP chemical shift in the direction of those of DHP-Pi. DHP and DHP-Pi can be thought of as two conformational extremes, with DHPs74e between these two extremes but much closer to the DHP-Pi conformation.

The DHPs74e structure shows that the addition of the negative charges at the end of the C-terminal helix does not cause much conformation change in the C-terminal subdomain. Instead, it is the orientation and conformation of the N-terminal subdomain that are dramatically altered. Based on the observed structure, we hypothesize that, after phosphorylation at Ser74, the phosphate group attracts Lys24 in the V-loop, which is mobile in solution (Figure 4B). As a result of the electrostatic attraction, the N-terminal loop moves much closer to the C-terminal helix and folds into a conformation that allows new hydrophobic interactions involving residues Val20, Val21, and Leu29, which become incorporated into the hydrophobic core. These new hydrophobic contacts further stabilize that conformation and induce adjustment in the entire N-terminal subdomain, giving rise to a ~25º orientation change of the C-terminal subdomain, relative to the V-loop (Figure 4C). Our relaxation data indicate that the V-loop in DHPs74e is still more mobile than the rest of the protein; however, the difference is less than that of the V-loop in DHP.

In the study of conformation changes in phosphorylated nitrogen regulatory protein C (NtrC), Kern and coworkers proposed the concept that NtrC is in equilibrium with two conformation states, while the phosphorylation shifts the equilibrium toward the active state (Kern et al., 1999; Volkman et al., 2001). The structural evidence suggests that DHP and DHP-Pi are in a very similar dynamic situation. It is likely that the V-loop of dematin headpiece is in equilibrium with a variety of conformation states, while phosphorylation stabilizes one conformation and shifts the equilibrium toward that folded state. The Ser-to-Glu mutation induces the conformational equilibrium shift in the same direction, yet the equilibrium constant may be slightly lower than that induced by phosphorylation. This is probably because at pH 6.0 the phosphate group carries one and a half negative charges distributed among three oxygen atoms (Andrew et al., 2002). In contrast, the carboxyl group in glutamic acid has only one negative charge distributed between two oxygen atoms. The Ser-to-Glu mutation does not change the overall fold of the actin binding motif in DHP, which explains why DHP-Pi still binds F-actin, although with an approximately 3-fold reduced affinity (Azim et al., 1995). The major characteristics of the charged crown motif are maintained, albeit with some minor side chain rearrangements (Figure 4B). These changes include residues Arg66, Arg49, Glu56, and Lys65, which do not alter the folding of the three α helices in the C-terminal subdomain, but may account for the decreased actin binding affinity of DHP-Pi.

The DHPs74e structure provides structural information about the phosphorylation-mediated actin bundling activity of dematin. Phosphorylation at Ser74 induces conformation changes of the V-loop in dematin headpiece and changes the dynamics of the loop as well. The rearrangement of the N-terminal loop could alter the orientation of the headpiece domain relative to the N-terminal core domain of dematin, thereby making the two actin binding sites unfavorable for F-actin bundling. Alternatively, if the N-terminal core domain and headpiece domain are connected by a flexible loop, facilitating easy bundling, then when dematin is phosphorylated, the headpiece domain may be locked in a specific orientation relative to the N-terminal core domain, which significantly limits the chances for bundling.
The relaxation experiments suggest a change in the mobility of the N-terminal loop in DHP, although the overall mobility of the N terminus is still higher than the rest of the protein. The conserved C-terminal location of all reported headpiece domains may indicate that the headpiece should not be tightly locked with the N-terminal core domain to fully exert its function. Another level of complexity derives from the trimeric nature of dematin in erythrocytes. The cyclic AMP-dependent protein kinase abolishes the actin bundling activity of both dematin monomer and trimer (Husain-Chishti et al., 1988). Since a dematin trimer should, in theory, contain six actin binding sites, both actin bundling and phosphorylation-regulated inhibition could only be achieved when the three subunits work in a synergistic manner. The headpiece domain has been proposed to be indispensable in dematin trimer formation (Khanna et al., 2002). It is possible that phosphorylation on one subunit of dematin not only inhibits the actin binding of that subunit, but induces a synergistic conformational change in other subunits as well. A complete understanding of the mechanism of kinase regulation on dematin awaits more structural information of the N-terminal core domain as well as the further physiological interpretation of dematin function and regulation.

Experimental Procedures

Site-Directed Mutagenesis, Protein Purification, and Phosphorylation

DHP, encoding residues 316–383 of the 48 kDa form of human dematin headpiece, was cloned in the plasmid pD48 (Vardar et al., 2002), to generate the s74e mutation, Ser381 was mutated to Glu by using QuikChange Site-Directed Mutagenesis (Stratagene). Prior to expression, the construct was sequenced at the Boston University Genetics Core facility. To facilitate the comparison with previous work, we use the amino acid numbering scheme reported by Vardar et al. (1999), by which residues 1–76 of DHP correspond to residues 308–383 of the 48 kDa form of human dematin and Ser381 becomes Ser74 in DHP. DHP and DHPs74e were expressed in Escherichia coli BL21 (DE3) cells (Novagen). Isotopic labeling was achieved in M97 minimal media supplemented with U-13C, U-15N, and U-2H labeled ammonium chloride and U-13C, U-15N, and U-2H labeled glucose (muchmore et al., 1989). The supernatant of the cell lysate was applied to a Sephadex G50 column, and fractions containing headpiece protein were further purified on a C18 reverse-phase column by high-performance liquid chromatography (HPLC). To generate the s74e mutation, Ser381 was mutated to Glu by using QuikChange Site-Directed Mutagenesis (Stratagene). Prior to expression, the construct was sequenced at the Boston University Genetics Core facility. To facilitate the comparison with previous work, we use the amino acid numbering scheme reported by Vardar et al. (1999), by which residues 1–76 of DHP correspond to residues 308–383 of the 48 kDa form of human dematin and Ser381 becomes Ser74 in DHP. DHP and DHPs74e were expressed in Escherichia coli BL21 (DE3) cells (Novagen). Isotopic labeling was achieved in M97 minimal media supplemented with U-13C, U-15N, and U-2H labeled ammonium chloride and U-13C, U-15N, and U-2H labeled glucose (muchmore et al., 1989). The supernatant of the cell lysate was applied to a Sephadex G50 column, and fractions containing headpiece protein were further purified on a C18 reverse-phase column by high-performance liquid chromatography (HPLC) (Vardar et al., 2002). The phosphorylation of Ser74 in DHP was catalyzed by cyclic AMP-dependent protein kinase (Sigma) as described previously (Frank et al., 2004). The extent and site of phosphorylation were determined by mass spectrometry (Boston University School of Medicine Mass Spectrometry Resource). Production of large amounts of phosphorylated DHP is hampered by insoluble products from phosphorylated DHP at additional sites as determined by mass spectrometry.

Multidimensional NMR Spectroscopy

All NMR samples were made in 20 mM sodium phosphate, 10% D2O, 0.02% sodium azide, with 1 mM 3-trimethylsilyl-tetradeutero sodium lactate (DHPs74e) or with presaturation of the water signal. The D溶液 concentrations varied from 1 to 4 mM depending on the protein availability and experimental requirements. All NMR spectra were acquired on a Bruker DMX500 spectrometer at 20°C unless otherwise stated. Water suppression was achieved with either the watergate pulse sequence (Pirollo et al., 1992) or with presaturation of the water signal. All 1H, 13C, and 15N chemical shifts were referenced against 1 mM TMSP (Wishart et al., 1995).

The following NMR spectra were collected for assignment and structure calculation: homonuclear two-dimensional DQF COSY; two-dimensional TOCSY (70 and 160 ms mixing time); two-dimensional NOESY (presaturation and watergate pulse sequence at 150 ms mixing time); two-dimensional NOESY in 100% D2O; heteronuclear two-dimensional 1H-N-HSQC (at 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C); heteronuclear two-dimensional 13C-HSQC; 1H three-dimensional NOESY (150 ms); 13C three-dimensional TOCSY; 13C three-dimensional HCC-HCOSY (Bax et al., 1990; Bodenheim and Ruben, 1989; Griesinger et al., 1988; Kay et al., 1993); triple resonance HNCA, HNCO, HN(CO)CA, and HN(CA)CO (Clubb et al., 1992; Grzesiek and Bax, 1992; Kay et al., 1994).

The backbone angular constraints were determined based on the JHN_w values, obtained from HNHA experiments (Forman-Kay et al., 1990; Vuister and Bax, 1993). The side chain γ torsion angle constraints were based on the intensity of crosspeaks in HNHB as well as on two-dimensional COSY and NOE spectra (Archer et al., 1991). Stereoscopic assignments for methyl groups in Leu and Val were achieved via a 13C-HSQC spectrum of a 10% U-13C-labeled, 100% U-15N-labeled sample (Neri et al., 1989).

Hydrogen bond constraints were based on amide hydrogen protection factors. A series of ~15 min 1H-NOESY spectra were collected immediately after lyophilized DHPs74e was dissolved in 100% D2O, at pH 6.0. The amide hydrogen exchange rates were determined by fitting the exponential decay function A e^-τ + C to the series of HSQC peak intensities of the individual residues by using NMRView (Johnson and Blevis, 1994). The protection factors were calculated by dividing the experimental exchange rates by the predicted rates for unfolded proteins calculated with the program SPHERE (Bai et al., 1993; Zhang, 1995).

All NMR data were processed with NMRPipe and NMRDraw (Delaglio et al., 1995) and were analyzed with NMRView (Johnson, 2004). A typical two-dimensional spectrum is an average of 32 or 64 transients of 512 × 2048 complex data points. A three-dimensional spectrum was typically acquired as an average of 4–64 transients of 64 × 128 × 1024 complex data points. The data were normally zero filled to twice their original size and apodized with shifted sine bell functions, followed by baseline correction and Fourier transformation.

15N-Relaxation Measurements

The 15N-R1, R2, and heteronuclear NOE experiments were acquired as previously described (Frank et al., 2004) and the sample contained ~4 mM DHPs74e in 20 mM phosphate buffer (pH 6.0), 20°C at 500.13 MHz with sensitivity enhanced pulse sequences (Farrow et al., 1994). The relaxation delays and order for the R1 experiment were 100, 5, 1000, 10, 50, 25, 50, 100, 10, and 100 ms. For the R2 experiment, the relaxation delays and order were 17.6, 70.4, 35.2, 52.8, 70.4, 105.6, 140.8, 211.2, 352.1, 17.6, and 70.4 ms. For the heteronuclear NOE experiment, the delay between scans was 5 s. For the NOE experiment, 120° pulses separated by 5 ms delays were incorporated into the relaxation delay to provide saturation. The NOE and noNOE experiments were acquired simultaneously in consecutive scans within one experiment. The R1 and R2 rates and the heteronuclear NOE values and their associated errors were determined from the peak intensities by using the fitting routines in NMRView (Johnson and Blevis, 1994).

Structure Calculations

A total of 1289 nonredundant NOE constraints were obtained from two-dimensional NOESY experiments in H2O or 100% D2O. NOE crosspeaks were classified as weak (1.8–5.0 Å), medium (1.8–3.8 Å), or strong (1.8–2.8 Å), based on their intensities. An additional 0.4 Å was added on all ambiguous assignments, including nonstereo-specifically assigned methylene, aromatic and methyl hydrogens. Another 0.5 Å was added for overlapped peaks or peaks close to water signals or other artifacts.

The backbone φ angles of 23 residues were constrained according to the J_HN, HN-NOE values, determined in an HNHA experiment. Another 39 φ angle constraints were included based on the Hα, 15N, 13Cα, and 13CO chemical shifts by using the TALOS prediction program (Cornilescu et al., 1999). The angle constraints derived from the TALOS prediction were only included after the initial model had been built. Those predictions not in agreement
with the initial model were excluded in the calculation unless further refined models validated those predictions. The error margin suggested by TALOS was applied for most constraints. The \( \phi \) angles predicted by TALOS in most cases are in close agreement with the values derived from \( \Delta\delta_{HN,\text{HN}} \) values. There were a total of 26 \( \phi \) angles constrained to \( \pm 40^\circ \) included in the structure calculations. These constraints were based on the stereospecific assignments of \( H_\alpha \) and the HNHB spectrum in conjunction with the two-dimensional DQFCOSY and NOESY data.

A total of 9 hydrogen bond constraints, giving rise to an additional 18 distance constraints, were included on the basis of the high amide hydrogen protection factors and the presence of NOE cross-peaks between \( H_\alpha \) and \( H_{\text{N}} \) of residues i to i + 3 and i to i + 4 (Wagner et al., 1987). The \( O_i \) to \( H_{\text{N}+4} \) distance was constrained to less than 2.3 \( \AA \), and the \( O_i \) to \( H_{\text{N}+5} \) distance was constrained to 2.5-3.3 \( \AA \).

The distance and angular constraints were used as inputs for distance geometry and simulated annealing starting from an extended strand conformation by using the software package CNS (Brunger et al., 1998). The simulated annealing was performed from 2500 K in 3000 high-temperature dynamics steps and 10,000 slow-cool annealing steps, by using the default scale factors for NOE and dihedral angle and van der Waals repulsions. After 100 cycles of calculations, 35 structures were accepted with no NOE violation larger than 0.5 \( \AA \) or dihedral angle violations larger than \( 5^\circ \). The 20 lowest-energy structures were used to calculate the minimized average structure.

Molecular Display and Structure Validation

The structures were displayed and analyzed with MOLMOL (Koradi et al., 1996). Distance measurements and surface potential calculations were performed in MOLMOL as well. All 20 structures were validated with the PROCHECK-NMR and AQUA software packages (Laskowski et al., 1996). Statistics for the structures are reported in Table 1.

Supplemental Data

Supplemental Data including a figure of the \( ^{15}\text{N}-\text{HSQC} \) spectrum with assignments and a figure showing a comparison of the number of NOE cross-peaks in DHP versus DHP74e are available at http://www. structure.org/cgi/content/full/14/2/379/DC1/.

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References


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The structural coordinates of DHPs74e have been deposited in the Protein Data Bank with accession code 1ZV6.