The NMR Structure of Dematin Headpiece Reveals a Dynamic Loop That Is Conformationally Altered upon Phosphorylation at a Distal Site*

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Dematin (band 4.9) is found in the junctional complex of the spectrin cytoskeleton that supports the erythrocyte cell membrane. Dematin is a member of the larger class of cytoskeleton-associated proteins that contain a modular “headpiece” domain at their extreme C termini. The dematin headpiece domain provides the second F-actin-binding site required for in vitro F-actin bundling. The dematin headpiece is found in two forms in the cell, one of 68 residues (DHP) and one containing a 22-amino acid insert near its N terminus (DHP+22). In addition, dematin contains the only headpiece domain that is phosphorylated, in vivo. The 22-amino acid insert in DHP+22 appeared unstructured in NMR spectra; therefore, we have determined the three-dimensional structure of DHP by multidimensional NMR methods. Although the overall three-dimensional structure of DHP is similar to that of the villin headpiece, there are two novel characteristics revealed by this structure. First, unlike villin headpiece that contains a single buried salt bridge, DHP contains a buried charged cluster comprising residues Glu\textsuperscript{39}, Arg\textsuperscript{66}, Lys\textsuperscript{70}, and the C-terminal carboxylate of Phe\textsuperscript{76}. Second, \textsuperscript{15}N relaxation experiments indicate that the longer “variable loop” region near the N terminus of DHP (residues 20–29) is dynamic, undergoing significantly greater motions that the rest of the structure. Furthermore, NMR chemical shift changes indicate that the conformation of the dynamic variable loop is altered by phosphorylation of serine 74, which is far in the sequence from the variable loop region. Our results suggest that phosphorylation of the dematin headpiece acts as a conformational switch within this headpiece domain.

Villin-type headpiece proteins are cytoskeleton-associated proteins that have a small ~76-amino acid “headpiece” domain at the extreme C terminus of a much larger “core” domain. Most, but not all, headpiece domains are modular F-actin-binding motifs that retain binding activity when cleaved from the core domain (1). Headpiece domains were originally identified in the protein villin, an F-actin cross-linking protein found in the actin bundles that support the brush border membrane of the absorptive epithelium (2, 3). Headpiece-containing proteins have since been found in a variety of tissues and cellular locations, suggesting a wide range of functions for this heterogeneous class of actin-binding proteins. The core domains of headpiece-containing proteins vary widely, often containing domains homologous to other actin-binding proteins. In contrast, headpiece domains have a unique sequence conserved among the ~25 known examples (4).

Dematin (band 4.9) headpiece is found at the extreme C terminus of the unique core domain of dematin (5, 6). In erythrocytes, dematin is located at the vertices of the hexagonal spectrin filament network and may serve as an additional anchor to fix the spectrin-actin complex with respect to the membrane (6). Northern blot analysis has shown that dematin is also found in skeletal muscle, heart, brain, kidney, and lung tissue.

Recently, a dematin headpiece knockout mouse was shown to have a fragile red cell phenotype with spheroidal erythrocytes, indicating an important role for the headpiece of dematin in maintaining the mechanical properties of the red cell membrane (7). Dematin has been mapped to human chromosome 8p21.1 (8), a region often deleted in prostate cancer. Indeed, aberrant expression of dematin leads to altered cell morphology, and overexpression of dematin results in a reversion of the tumorigenic PC-3 cell line to that of normal prostate cells (9). Dematin forms functional trimers in the erythrocyte (10). Each trimer contains two 48-kDa units and one 52-kDa unit. The difference in these two forms is due to alternate RNA splicing within the headpiece domain, where the 52-kDa isoform contains an additional 22 amino acids near the headpiece N terminus (8). The 22-amino acid insert contains an 11-residue sequence homologous to band 4.2 (12). Whereas the role of this phosphorylation in vivo...
is unknown, phosphorylation of dematin inhibits in vitro F-actin bundling but not F-actin binding (1, 13).

The headpiece domain of villin has been the most intensely investigated headpiece domain (1, 3, 14–16). The NMR structures of villin headpiece (HP67) and a small subdomain within villin headpiece (HP35) reveal a unique two-domain structure (4, 17). The N-terminal half of villin headpiece has little regular secondary structure and its folding is pH-dependent due to a buried histidine residue. The C-terminal half folds to form a three helix motif that is pH independent and forms a stable subdomain. The two domains are held together by a buried salt bridge. A comparison of the known headpiece sequences with the NMR structure also suggests a loop in the N-terminal domain that varies in length between headpiece sequences. This variable length loop (V-loop) has been proposed to be a general feature of headpiece domains. The putative 10 residue dematin headpiece V-loop is the longest within any headpiece sequence. Although the NMR structure of HP67, in conjunction with mutagenic data, suggests the features that are critical for F-actin binding (1, 4) no other headpiece domain structure has been determined to test the validity of the generalization made from villin headpiece on this class of sequences.

The NMR structure of the 68-residue dematin headpiece (DHP), corresponding to the 48-kDa form of dematin headpiece, reported here reveals that whereas the overall structure and actin-binding surface are similar to those of villin headpiece, there are significant differences in the V-loop region and in the buried salt bridge between the N- and C-terminal subdomains. The DHP V-loop region is longer, as expected, but is also dynamic and therefore less well defined than in the HP67 NMR structure. Interestingly, the chemical shifts within the V-loop region are extremely sensitive to the phosphorylation state of serine 74 which is some 40 residues away in the sequence, whereas the chemical shift changes near serine 74 are slight, indicating little change in structure at the phosphorylation site. The buried salt bridge in HP67 between glutamic acid 39 and lysine 70 is not retained in DHP, but is replaced by a salt bridge cluster composed of glutamic acid 39, arginine 66, lysine 70 and the C-terminal carboxylate of phenylalanine 76.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Dematin headpiece without (DHP) or with the 22-amino acid insert (DHP+22) was expressed from the plasmid pD48 or pD52, respectively, in Escherichia coli and purified as previously described (1). Isotopically enriched preparations were grown in minimal media supplemented with [15N]labeled ammonium chloride and/or [13C]-labeled glucose. The DHP construct spans residues 32–383 of the 48-kDa form of human dematin. For the complete sequences, see Fig. 5A.

Purified DHP was phosphorylated at serine 74 as previously described (13). Briefly, 10 mg of [15N]-labeled DHP was incubated with 2500 units of protein kinase A catalytic subunit (Sigma) in 20 mM tris(hydroxymethyl)aminomethane, pH 8.0, 50 mg/ml dithiothreitol, 1 mM ATP, 2 mM MgCl2, and 0.7 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid for 5 days at 30°C. Approximately one-third of the DHP was phosphorylated. The reaction was followed by reverse phase high performance liquid chromatography (HPLC) using C18 columns (Vydac) and water/acetonitrile gradients containing 0.1% trifluoroacetic acid.

To facilitate the comparison with other headpiece domains, we have used the numbering scheme from Vardar et al. (4), such that the N-terminal proline of DHP is residue 9, and C-terminal phenylalanine is residue 76. The final residue (Phe) is the natural C-terminal residue of dematin. The molecular weights of the purified proteins were verified by mass spectral analysis (Boston University School of Medicine Mass Spectrometry Resource).

Histidine pK Measurements—One-dimensional NMR spectra of a 1 mM sample in 100% D2O were acquired over a range of pH values from 1.9 to 7.2 at ~0.25-pH unit intervals. No correction was made for the deuterium isotope effect on the readings of the pH meter. The change in the chemical shift of the histidine indole H2, which at each pH was fit to a simple sigmoidal curve to determine the pK.

Multidimensional NMR Spectroscopy—NMR sample concentrations ranged from 0.7 to 4 mM protein. NMR samples contained ~0.5 mM 3-trimethylsilyl tetradecuto sodium propionate as a chemical shift reference, 10 mM phosphate, pH 6.0, 1.0% D2O, and 0.01% sodium azide, otherwise specified. The pH was adjusted to 7.0 using NaOH without correction for the effect of the D2O on the measured pH value.

NMR spectra were acquired on a Bruker DMX500 spectrometer. Water suppression was achieved with the watergate pulse sequence (18) or with presaturation of the water signal. All 1H, 13C, and 15N chemical shifts are referenced against 0.5 mM 3-trimethylsilyl tetradecuro sodium propionate (19).

NMR spectra for assignment and structure determination at pH 6.0, 20°C, included homonuclear two-dimensional NOEY (50-, 75-, 100-, 125-, and 150-ms mixing times), two-dimensional NOEY in 100% D2O (150 ms), two-dimensional TOCSY (20, 40, 50, 70, 80, and 110 ms), and two-dimensional DQFCOSY spectra. Heteronuclear experiments included 15N HSQC. 13N three-dimensional NOEY (150 ms), two-dimensional TOCSY, 13C HSQC, 13C three-dimensional NOEY (150 ms), and HCCH COSY (20–23). Triple resonance experiments for assignment included HNCA, HN(CO)CA, HNCO, and HN(CA)CO spectra.

Three-dimensional spectra were acquired as the average of 32 or 64 transients. Three-dimensional spectra were typically acquired as the average of 4–64 transients of 64 × 128 × 512 complex data points. The data were typically zero-filled or linear predicted to twice the original size, followed by apodization with shifted sine bell functions, prior to Fourier transformation and base-line correction.

Amide Hydrogen Exchange—Amide hydrogen exchange rates were measured in a series of 13N HSQC spectra after the addition of 100% D2O to a lyophilized, 15N-enriched NMR sample at pH 6.0. The spectra were acquired using pre- saturation of the small residual water signal. The total data points acquired were 2048 × 128 × 512 complex data points. The data were typically zero-filled or linear predicted to twice the original size, followed by apodization with shifted sine bell functions, prior to Fourier transformation and base-line correction.

13N Relaxation Measurements—R1, R2, and heteronuclear NOE data were acquired at 20°C, pH 6.0, at 500.13 MHz, with sensitivity-enhanced NOE experiments (24). Relaxation delays and order for the R1 experiments were 20, 60, 141, 236, 372, 753, and 1115 ms. For the R2 experiment, the delays and order were 24, 39, 55, 79, 110, 141, 251, 8, 55, and 8 ms. For the heteronuclear NOE spectra, the total relaxation delay was 5 s, with presaturation during the final 3 s for the NOE spectrum. The NOE and NOE reference spectra were acquired in continuous scans during one measurement. The R1 relaxation rates and heteronuclear NOE values and their associated errors were determined from the peak intensities using the routines in NMRView.

Structure Calculations—The restraints used for structural calculations and the structural statistics are summarized in Table I. The two-dimensional NOE build-up curves (from 50- to 150-ms mixing times) were fitted to a linear up to 150 ms. Expectations using heteronuclear distance restraint lists were the 13N three-dimensional NOEY (150-ms mixing time), two-dimensional NOEY (150-ms mixing time), and two-dimensional NOEY in 100% D2O (150 ms mixing time). NOE cross-peaks were classified as weak (1.8–4.8 Å), medium (1.8–3.2 Å), and strong.
packages (41, 42). The 12 lowest energy structures were sub-
calculated with MOLMOL. The 12 lowest energy structures were dis-
played and analyzed using the molecular graphics program MOLMOL (40). Surfaces and surface potentials were also calculated with MOLMOL. The 12 lowest energy structures were used to calculate a minimized average structure. The coordinates of the minimized average structure and those of the 12 lowest energy structures have been submitted to the Protein Data Bank.

The backbone ϕ angles of 20 residues were constrained based on the 3JHN,Cα values measured in an HNHA spectrum. The Ha, Ca, and Cß chemical shifts were used as input for the program TALOS (37) to provide an additional 22 ϕ restraints as well as 37 ψ restraints. The angles were restrained to the output stipulated by TALOS with error margins of ±30° except for two angles where TALOS predicted 30° ≤ error ≤ 40°, and the error margin was restrained to ±40°. The restraints from TALOS were consistent with the 3JHN,Cα coupling constants determined from HNHA and HMQC-J spectra. There were a total of 13 χ1 angles constrained with error margins of ±40° derived from the HNHB spectrum and inspection of the short mixing time NOESY data.

The identification of 10 hydrogen bonds allowed the addition of 20 distance restraints. These hydrogen bonds were identified on the basis of 1) protection from exchange as evidenced by the hydrogen exchange experiment and 2) the presence of Hα HN NOESY cross-peaks between residues i to i + 3 and i to i + 4 (38). For each hydrogen bond, the amide hydrogen to the oxygen distance was restrained to less than 2.3 Å, and the amide nitrogen to oxygen distance was restrained to the range 2.5–3.3 Å.

The distance and angle restraints were used as input for distance geometry followed by dynamic simulated annealing regularization and refinement using the software package CNS (39). The simulated annealing for each structure was carried out from 2500 K to a final temperature of 100 K over 25,000 steps. The 12 lowest energy structures were used to calculate a minimized average structure. The coordinates of the minimized average structure and those of the 12 lowest energy structures have been submitted to the Protein Data Base.

**Molecular Display and Surface Potential Calculation**—Calculated structures were displayed and analyzed using the molecular graphics program MOLMOL (40). Surfaces and surface potentials were also calculated with MOLMOL. The 12 lowest energy structures were subjected to validation by the PROCHECK-NMR and AQUA software packages (41, 42).

**RESULTS**

We expressed and purified both of the dematin headpiece constructs DHP and DHP++ (which contains the inserted sequence near the N terminus). The NMR sample of the DHP construct at 20 °C, pH 6.0, gave the high quality 15N HSQC spectrum shown in Fig. 1B with well dispersed peaks with little overlap, as expected for a well folded monomeric, 68-residue polypeptide. The HN residue of Gly10 is the only missing resonance from this spectrum and is observable only at 10 °C. On the other hand, the data from the DHP++ construct displayed an 15N HSQC spectrum nearly identical to that of DHP with additional peaks, falling into three regions in the spectrum (circled in Fig. 1A). These new peaks, arising from the 22-amino acid insert sequence have very low dispersion, with values between 8.1 and 8.8 ppm, consistent with a predominately unfolded structure for the insert sequence. From the high correspondence of the disperse peaks in the DHP++ spectrum with the DHP spectrum and the low chemical shift dispersion of the new peaks from the 22-amino acid insert, we conclude that the insert sequence in DHP++ is unstructured.

Additional peaks outside the region shown have no overlap, as expected for a well folded monomeric, 68-residue polypeptide. The HN residue of Gly10 is the only missing resonance from this spectrum and is observable only at 10 °C. On the other hand, the data from the DHP++ construct displayed an 15N HSQC spectrum nearly identical to that of DHP with additional peaks, falling into three regions in the spectrum (circled in Fig. 1A). These new peaks, arising from the 22-amino acid insert sequence have very low dispersion, with values between 8.1 and 8.8 ppm, consistent with a predominately unfolded structure for the insert sequence. From the high correspondence of the disperse peaks in the DHP++ spectrum with the DHP spectrum and the low chemical shift dispersion of the new peaks from the 22-amino acid insert, we conclude that the insert sequence in DHP++ is unstructured.

**An Unprotonated Histidine Residue in the Core of DHP**—To determine whether the N-terminal domain of DHP, like villin headpiece, contains an unprotonated histidine buried in the hydrophobic core, the pKₐ of His41 was measured by one-di-

dimensional NMR. The change in the chemical shift of the side chain histidine Hε2 atom as function of the measured pH in 50% D₂O is shown in Fig. 2. The measured pKₐ of His41 in DHP (pKₐ = 4.6) is 1.4 pH units below that of free histidine (pKₐ = 6.0). This shift is 0.4 pH units greater than that for villin headpiece (pKₐ = 5) observed previously (4), suggesting a more stable N-terminal subdomain structure for DHP than that of villin headpiece.

**NMR Structure of Dematin Headpiece**—The structure of DHP was calculated using a combined distance geometry/simulated annealing approach with the CNS software package (39). Of the 50 calculated structures, 35 were accepted with no angle violations greater than 5° and no NOE distance viola-
tions greater than 0.3 Å. The 12 lowest energy structures were used to calculate a minimized average structure. The structural statistics for the 12 lowest energy structures are pre-

![Fig. 1. 15N HSQC spectra of DHP+22 (A), DHP (B), and DHP-P (C) at pH 6.0, 20 °C. Additional peaks outside the region shown have no significant chemical shift differences between spectra. Backbone and side chain NH resonances are labeled by residue number in the spectrum of DHP (B). The HN peak of Gly10 is folded into this spectrum about the 15N dimension, and its position is indicated with a circle. The circles in A indicate the position of most of the resonances arising from the 22-amino acid insert in DHP+22. The lines in C indicate chemical shift changes relative to DHP (B) for a few disperse peaks in the region of the V-loop resulting from phosphorylation.](https://example.com/fig1.png)
of 2H2O on the measured pD values. See function of the measured pD. No correction has been made for the effect chemical shift of the H2 ring proton of His 41 at 20 °C.

Experimental details. For comparison, the data from the p

highly compact and is largely able despite the low backbone r.m.s. deviation between the structure. However, the presence and/or location of 3-10 helical structure displays two regions classified as 3-10 helix (residues 35–37 and 39–41) near the center of the sequence and the structure. However, the presence and/or location of 3-10 helical structure in the individual accepted structures is highly variable despite the low backbone r.m.s. deviation between the structures in this region (0.09 Å). The C-terminal subdomain is highly compact and is largely α-helical, with three helices spanning residues 44–50, 55–60, and 64–73.

The residues that make up the hydrophobic core from the N-terminal subdomain (Fig. 3C) are Tyr14, Tyr16, Leu19, Pro20, Val23, Leu24, Glu29, His41, and Leu42. Histidine 41 is a key component of this hydrophobic core, with 0.1% solvent-accessible surface area, and has extensive contacts to Val23 and Tyr14. The residues that make up the hydrophobic core from the C-terminal subdomain are Phe27, Phe31, Phe38, Arg45, Asn57, Leu69, Lys70, Ala71, and Leu75.

A Buried Charged Cluster in DHP—Unlike the structure of villin headpiece (HP67) that has two buried residues forming a salt bridge (Glu39 and Lys40), DHP has 3 charged residues buried in the hydrophobic core: Glu39, Arg45, and Lys70, which are all less than 10% solvent-exposed. In addition, the charged side chain groups of each of these residues and the C-terminal carboxylate group of Phe76 are all in close proximity to one another (Fig. 3D). The negatively charged carboxyl group in Glu39 appears to interact with the positively charged amino group of Arg45 and Lys70. This is in contrast to HP67, where the salt bridge is to Lys35. In the DHP NMR structure, Lys70 is also close to the carboxylate of the C-terminal Phe27, and we believe that it may use this carboxylate to neutralize the positive charge on the side chain of lysine 70. In most of the calculated structures, these charges were within 5 Å of one another.

Comparison with the Villin Headpiece Structure—The NMR structure of DHP is similar to that of villin headpiece (HP67), and the backbones align with a 1.9 Å r.m.s. deviation (Fig. 4A). DHP contains a backbone-backbone hydrogen bond, a motif shared with HP67, between Tyr14 and Leu42, which have extensive NOE contacts. Save for the variable loop, HP67 and DHP have very similar N-terminal structures, with an r.m.s. deviation of 1.55 Å for residues Gly10-Val20, Lys20-Leu42 in HP67 and DHP, and Lys35-Val22, Lys20-Leu42 in HP67. The three C-terminal residues 74–76 are well defined in both the DHP and HP67 NMR structures.

The conformation of the three C-terminal helices in the DHP NMR structure is almost identical to the conformation of those in the HP67 structure and, to a lesser extent, those in the HP35 subdomain (Fig. 4A). An alignment of the backbone for residues 42–76 between the minimized average structures of HP67 and DHP yields an r.m.s. deviation of 1.28 Å. Excluding the three C-terminal residues that are disordered in HP35, the backbone r.m.s. deviation of DHP and HP35 (residues 42–73) is 1.59 Å. The position of the conserved, buried hydrophobic residues in the C-terminal subdomain (Phe27, Phe31, Phe38, and Leu40) are also in close agreement among all three structures. Thus, the structure of the C-terminal subdomain is highly conserved.

Features of the F-actin Binding Face—Several residues important for actin-binding by villin headpiece have been identified by a combination of mutagenesis and peptide binding (14) and by cysteine-scanning mutagenesis (16). When those residues are mapped onto the NMR structure of HP67 (4), they reveal a hydrophobic “crown,” an alternating charged “crown,” and a positively charged “patch” (Fig. 4B).

Examination of the charged potential surface of the same putative actin-binding face of DHP reveals the retention of the hydrophobic cap, the positively charged patch, and, to a lesser extent, the charged crown. Trp35, the component of the hydrophobic cap in both HP67 and DHP is absolutely conserved among all headpiece domains. The basic patch in HP67 is comprised predominantly from the amino group Lys20, whereas the analogous basic group in DHP is from Arg45. The charged crown in HP67 is made primarily from residues Lys35, Lys40, Glu72 and the carboxylate group of Phe76 (4). In DHP, the residues that make up the alternating charged crown are Glu69, Lys72, Lys71, and the carboxylate group of Phe76. The

A Buried Charged Cluster in DHP
charge pattern is similar except that the dematin alternating charged crown is at a steeper angle, and there is significantly more positive charge throughout the surface.

**A Dynamic V-loop in DHP**—The largest difference between the DHP and HP67 NMR structures is in the V-loop, residues Val^{20}–Lys^{29}, which is 2 residues longer in DHP than in HP67. The V-loop in the DHP NMR structure is more solvent-exposed and less well ordered than in the HP67 structure. None of the residues in the DHP V-loop are significantly protected from backbone amide hydrogen exchange.

To differentiate whether the poor agreement of calculated NMR structures in the region of the V-loop was the result of a lack of NMR restraints or due to actual disorder, we performed $^{15}$N-relaxation experiments on $^{15}$N-labeled DHP. The R1, R2,
and $^{15}$N-$^1$H heteronuclear NOE values are plotted versus the sequence and compared with the backbone r.m.s. deviations of the calculated structures in Fig. 5. The R1 values are uniform throughout the sequence. However, there are significant differences in the R2 values for residues 20–29 (the V-loop region). There are both increases and decreases in R2 in the V-loop region relative to the average in R2. There are also decreases in the $^{15}$N-$^1$H heteronuclear NOE values in the V-loop. These changes in R2 and heteronuclear NOE values are consistent with a greater degree of flexibility in the V-loop of dematin headpiece.

A Change in the Conformation of the V-loop upon Phosphorylation of Ser$^{74}$—Dematin is the only headpiece domain that is phosphorylated, in vitro, at serine 74. To access the effect of phosphorylation on the structure of DHP, we phosphorylated Ser$^{74}$ with the catalytic subdomain of protein kinase A to produce DHP-P. The DHP-P was then separated from unreacted DHP by HPLC. In the $^{15}$N HSQC spectrum of DHP-P (Fig. 1C), several resonances have altered chemical shifts compared with the $^{15}$N HSQC spectrum of DHP. Surprisingly, the region with the largest changes in chemical shifts is the V-loop region (Fig. 6) and not around the site of phosphorylation. Indeed, the chemical shifts of some resonances, particularly in the most dynamic portion of the V-loop, are so large that it is impossible to track their new positions on the spectrum by simple inspection. However, this strategy can be used to assign the chemical shift changes in the more disperse peaks that flank the V-loop, which show moderate chemical shift perturbations. Thus, phosphorylation near the C terminus of DHP results in a large conformational change in the V-loop residues (residues 20–29) and to a lesser extent in the flanking region while producing virtually no conformation change at the actual site of phosphorylation.

DISCUSSION

Villin-type headpiece domains are structurally unique motifs that often, but not always, function as F-actin targeting/bind-
these two C-terminal residues are well ordered, whereas the N-terminal residues are unstructured. Unstructured N and C termini are often seen in protein structures, and the high ordering of the C-terminal residues in dematin and villin headpiece suggests that their precise conformation is important for F-actin binding. This is further supported by the observation that in HP35 (the independent C-terminal subdomain of villin), Leu75 and Phe76 are disordered, and it cannot bind F-actin, although the structures are otherwise quite similar.

The V-loop of DHP is anchored to the rest of the structure by the incorporation into the hydrophobic core of Leu19 at the N-terminal side and Pro30 at the C-terminal side. These residues are structurally analogous to residues Leu21 and Pro30 in the HP67 structure. The amide of Val20 that marks the beginning of the V-loop is involved in a backbone hydrogen bond to Tyr16 in all of the calculated NMR structures. This hydrogen bond also assists in fixing the N-terminal end of the V-loop. The R2 and 15N-1H heteronuclear NOE values of Gly25, at the center of the V-loop, are comparable with those near the unordered N terminus of DHP, suggesting that the center of the V-loop is nearly as flexible as the unstructured N terminus.

Surprisingly, we find large changes in the chemical shifts of the amide protons within the V-loop upon phosphorylation of Ser74, indicating a significant change in the conformation. Furthermore, there are only small changes in the chemical shift of Ser74 and its neighboring residues, suggesting that the confor-

**FIG. 6.** Phosphorylation induces chemical shift changes in the DHP V-loop. The difference in backbone amide hydrogen chemical shifts in the HSQC spectra of DHP-P (Fig. 1C) and DHP (Fig. 1B) are plotted against the DHP sequence. The asterisks indicate positions where the chemical shifts cannot be readily made by comparison with the DHP spectrum.

A flexible V-loop in DHP. A, the sequence of DHP with the V-loop and five helices labeled. The sequence and location of the 22-amino acid insert is shown above the DHP sequence. B, the pairwise backbone r.m.s. deviation values for the 12 accepted DHP structures. C, the R1 relaxation rates. D, the R2 relaxation rates. E, the 15N-1H heteronuclear NOE experiment. See “Experimental Procedures” for experimental details. A horizontal line is drawn at the average value to highlight differences along the sequence.
NMR Structure of Dematin Headpiece

The NMR structure is relatively unaltered at the site of phosphorylation near the C terminus. The site of phosphorylation is some 44 residues from the V-loop in the amino acid sequence. However, the V-loop and Ser24 are in relatively close proximity in the three-dimensional structure. There are three positively charged residues in the V-loop that could interact with the negatively charged phosphate group to form a salt bridge: Lys26, Arg28, and Lys28. The side chain of Lys26 makes the closest approaches to the side chain of Ser24 in the accepted NMR structures, with distances ranging from 5.9 to 18.8 Å. The distances between the Ser24 side chain and those of Arg26 and Lys28 are always greater than 11 Å. The biological role of the phosphorylation of DHP has yet to be demonstrated, but phosphorylation results in a 3-fold reduction of the actin affinity of DHP (1). In the context of full-length dematin, phosphorylation results in loss of F-actin bundling activity but not F-actin binding. Further studies are required to determine the role of the phosphorylation-induced conformational change we have observed on the properties of intact dematin.

The NMR structure described here, the second structure of a villin-type headpiece domain, confirms the generality of several features of headpiece domains but also reveals new and unexpected features: a highly flexible V-loop region that becomes ordered upon phosphorylation near the C terminus and the change from a single buried salt bridge in villin headpiece to a buried charge cluster in dematin. Thus, whereas the overall structures of headpiece domains are likely to be similar, the details may be more diverse than expected from simple examination of their sequences.

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