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Phosphorylation of the minimal inhibitory region at the C-terminus of caldesmon alters its structural and actin binding properties

Valerie B. Patchell [a], Alexander V. Vorotnikov [b], Yuan Gao [c], Douglas G. Low [c,d], James S. Evans [c,1], Abdellatif Fattoum [e], Mohammed El-Mezgueldi [d], Steven B. Marston [d,*], Barry A. Levine [a,c]

[a] *School of Medicine, University of Birmingham, Birmingham B15 2TT, UK*
[b] *Institute of Experimental Cardiology, Cardiology Research Centre, Moscow 121552, Russia*
[c] *School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK*
[d] *Department of Cardiac Medicine, Imperial College School of Medicine at National Heart and Lung Institute, Dovehouse St., London SW3 6LY, UK*
[e] *CRBM/CNRS UPR 1086, 34293 Montpellier Cedex 5, France*

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Abstract

Caldesmon is an inhibitory protein believed to be involved in the regulation of thin filament activity in smooth muscles and is a major cytoplasmic substrate for MAP kinase. NMR spectroscopy shows that the actin binding properties of the minimal inhibitory region of caldesmon, residues 750–779, alter upon MAP kinase phosphorylation of Ser-759, a residue not involved in actin binding. This phosphorylation leads to markedly diminished actin affinity as a result of the loss of interaction at one of the two sites that bind to F-actin. The structural basis for the altered interaction is identified from the observation that phosphorylation destabilises a turn segment linking the two actin binding sites and thereby results in the randomisation of their relative disposition. This modulatory influence of Ser-759 phosphorylation is not merely a function of the bulkiness of the covalent modification since the stability of the turn region is observed to be sensitive to the ionisation state of the phosphate group. The data are discussed in the context of the inhibitory association of the C-terminal domain of caldesmon with F-actin.

Keywords: Caldesmon; Phosphorylation; Actin binding; Nuclear magnetic resonance; Mitogen-activated protein kinase

1. Introduction

The regulation of biochemical activity by reversible protein phosphorylation is a pervasive cellular control mechanism. Structural analyses of kinase-substrate complexes have provided much insight into the recognition of protein substrates and the mechanism of phosphoryl transfer, yet comparatively little is known about how phosphorylation modulates protein structure to effect the alteration of biological activity [1,2]. The purely physical changes that result from the introduction of a covalently bound phosphate group, such as altered steric and electrostatic characteristics and the ability to form hydrogen bonds, amply explain the effects observed in the

* Corresponding author. Fax +44-20-7823-3392.
E-mail address: s.marston@ic.ac.uk (S.B. Marston).
[1] On sabbatical leave from Lawrence University, Appleton, WI 54912-0599, USA.

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case of isocitrate dehydrogenase [3]. In most other instances, such as glycogen phosphorylase [4,5] and the cyclin-dependent protein kinase, CDK2 [6] for example, the phosphorylation-dependent conformational response is enabled by incorporation of the phosphoryl group into a pre-existing network of interactions within well defined tertiary structure [1,2,7]. There are also, however, examples where phosphorylation alters the structural and biological properties of peptides that possess little secondary or tertiary structure [8–10]. These observations suggest that the presence of a phosphate group exerts direct (local) effects as well as inducing indirect (global) changes of protein conformation. Thus although phosphorylation may alter target-interaction affinity through simple electrostatic changes, phosphorylation-induced structural changes may also alter or switch binding partners.

Caldesmon is an example of a relatively unstructured actin inhibitory protein that is phosphorylated by several kinases [11–13]. Our earlier studies on caldesmon showed that a minimal inhibitory peptide (Leu750-Trp779, LW30) contained short actin binding motifs at its N- and C-terminal ends linked by a partly structured segment. Of particular interest to the structural basis for inhibition and its regulation by phosphorylation is the extended *trans*-X-Pro sequence Ser_{759}-Pro-Ala-Pro-Lys-Pro_{764} that includes a MAP kinase phosphorylation site at Ser-759. Caldesmon LW30 peptide is therefore an ideal subject in which to study by NMR methods the direct effects of phosphoryl groups in the absence of tertiary interactions.

The ability of caldesmon to inhibit actomyosin ATPase activity involves the C-terminal 171 amino acids (domain 4: amino acids 622–793 of human caldesmon, 600–756 in the original chicken sequence [14–16]). Domain 4 interacts with actin through three short, non-sequential segments (C, B and B′) [17]. Peptide sequences that match only one of the actin binding segments do not, however, display inhibitory properties, suggesting that functional association with actin involves a combination of contacts on domain 4. Two of these contacts (termed B and B′) are located in the extreme C-terminus of caldesmon, designated domain 4b (residues 726–793 human), which forms a fully functional inhibitory domain with a highly conserved sequence [18–20]. The minimal inhibitory sequence has been identified in a 30 residue peptide Leu750-Trp779 (LW30) [21]. The LW30 peptide is capable of inhibiting actomyosin ATPase activity at low ionic strength and essentially comprises the two actin contact sites of domain 4b and the intervening sequence. This sequence includes part of site B (Leu_{750}-Thr-Lys-Thr), the extended *trans*-X-Pro sequence Ser_{759}-Pro-Ala-Pro-Lys-Pro which contains the MAPK phosphorylation site and a well defined β-turn motif $_{770}$Gly-Asp-Val-Ser_{773} near the second actin contact site (B′, Leu-Trp_{779}) [22]. The turn constrains the relative orientation of the two actin binding regions and potentially could bring sites B and B′ close together. This arrangement can explain the multipoint docking of caldesmon on actin.

As a starting point towards resolution of the structural consequences of caldesmon phosphorylation at Ser-759 we have used NMR to compare the influence of this single site modification in two model peptides: the LW30 peptide phosphorylated by MAP kinase and a short phospho-peptide, 755–765, DS11-P that contains just the sequence around Ser-759. We found that Ser-759 phosphorylation of LW30 induced long-range conformational effects that correlate with the loss of one of the two actin contacts and therefore could provide a structural explanation for the decrease in inhibitory function caused by phosphorylation.

2. Materials and methods

2.1. Preparation of LW30 and DS11-P

The 11 residue peptide, *N*-acetylDGNKS_PPAPKP-Samide, residues 755–765 (698–708 in the chicken sequence [15]), singly phosphorylated at the serine residue corresponding to Ser-759, was synthesised by Alta Bioscience (Birmingham, UK). The composition and purity of the peptide were confirmed by mass spectrometry and by 2D NMR analysis. The LW30 peptide comprising human caldesmon residues 750–779 (693–722 in chicken) was synthesised and purified as previously described [22].

2.2. MAP kinase phosphorylation of LW30

The His-tagged constitutively active MEK1ca was bacterially expressed from a plasmid kindly donated

by Dr. P.H. Sugden. 1 mg/ml recombinant GST-p44^{erk1} MAP kinase [23] was incubated for 2 h at 33°C with 0.05 mg/ml of MEK1ca in buffer A (20 mM HEPES (pH 8.0), 20 mM NaCl, 3 mM $MgCl_2$, 0.25 mM *o*-vanadate, 0.1 mM EGTA, 1 mM dithiothreitol) containing 0.4 mM MgATP. Imidazole (pH 7.9) was added to a final concentration of 5 mM and the mixture was passed through 1 ml of TALON resin to remove the His-tagged MEK1ca. The active MAP kinase was dialysed against buffer A and used for LW30 phosphorylation. 4.3 mg of LW30 (2.2 mg/ml, 660 μM) was incubated for 4 h at 32°C with 0.15 mg/ml (2.1 μM) of freshly activated GST-p44^{erk1} MAP kinase in the buffer containing 20 mM HEPES (pH 8.0), 20 mM NaCl, 2.5 mM ATP, 7.5 mM $MgCl_2$, 0.25 mM *o*-vanadate, 0.1 mM EGTA, 1 mM dithiothreitol. The stoichiometry of LW30 phosphorylation was determined from a shift of its electrophoretic mobility in continuous 10% polyacrylamide gel containing 8 M urea. The gel was run in 25 mM Tris/glycine (pH 8.6) from anode to cathode, fixed in 10% TCA and conventionally stained with Coomassie R250. The phosphorylated peptide product was isolated by HPLC. The purity of both phosphorylated and unphosphorylated peptides was confirmed by mass spectrometry and subsequent NMR analysis. The ^{1}H NMR spectra of each of the peptides at pH 4.8 and at pH 6.5 were invariant over the concentration range 0.1–5$\times10^{-3}$ M. The narrow line widths observed were taken to indicate the absence of aggregation.

2.3. NMR spectroscopy

Spectra were typically acquired using a 12 ppm sweep and 128 transients of 16K data points with presaturation of the solvent. For the assignment of resonances we used standard homonuclear 2D total correlation (TOCSY), nuclear Overhauser effect spectroscopy (NOESY) experiments (250 and 500 ms mixing time) and rotating frame Overhauser effect spectroscopy (ROESY) experiments (200 ms mixing time). Samples for 2D experiments typically comprised 10^{-3} M peptide dissolved in 90% H_2O/10% ^2H_2O, pH 4.8 and pH 6.5. Spectra were acquired at 285 K using either 600 MHz (Varian Unity+) or 500 MHz (Bruker) instruments. Binding titrations with F-actin, pH 7.6, 285 K and peptide concentrations of 0.4$\times10^{-3}$ M were carried out at 500 MHz by addition of small aliquots of a 10 mg/ml solution of the protein as previously described [22].

3. Results

3.1. The localised changes associated with phosphorylation of Ser-759

Comparative NMR study of the two phosphorylated caldesmon peptides of different residue length, LW30 and DS11-P, was undertaken so as to distinguish between localised and long-range effects resulting from the modification of Ser-759. In order to do this we developed a technique which allows us to obtain 10–20 mg of MAP kinase phosphorylated LW30 peptide (inset, Fig. 1). Both LW30-P and DS11-P peptides were studied over the pH range 4–7.5 in order to investigate any influence of phosphate ionisation. The backbone amide signal of the phosphorylated serine residue was clearly resolved downfield of the remaining –NH resonances (Figs. 1 and 2A). This enabled the derivation of the apparent pK_a of the phosphate group, 5.5 ± 0.2 for both peptides, obtained from the chemical shift variation of the amide proton of Ser-759P ($\Delta\delta$ = 0.46 ± 0.08 ppm) (Fig. 2). In the case of the shorter DS11-P peptide (Fig. 3), signals of the other residues corresponded to those previously found for unphosphorylated LW30 [22] and showed little or no variation with pH. Only the signals of residues of DS11-P immediately adjacent to the phosphorylated serine showed detectable (0.02–0.05 ppm) sensitivity to the phosphate pK_a indicative of only localised shielding effects of phosphate ionisation for DS11-P, residues 755–765.

A *trans* peptide backbone conformation for each X-Pro pair in the sequence –S$_P$PAPKP– of DS11-P was indicated by the characteristic sequential proton NOE proximities ($X_{H\alpha}$-Pro_δ) and the prescriptive downfield shift (approx. 0.25 ppm) of the –CH_α resonance of each of the residues preceding proline (Fig. 3) [24]. Non-sequential NOE were not observed which indicates the lack of any other conformational preference over the pH range studied. The same local effects and extended *trans*-X-Pro conformation was observed in the longer LW30 peptide phosphorylated by incubation with MAP kinase. Resonance assign-

750LTKTPDGNKSPAPKPSDLRPGDVSGKRNLW779

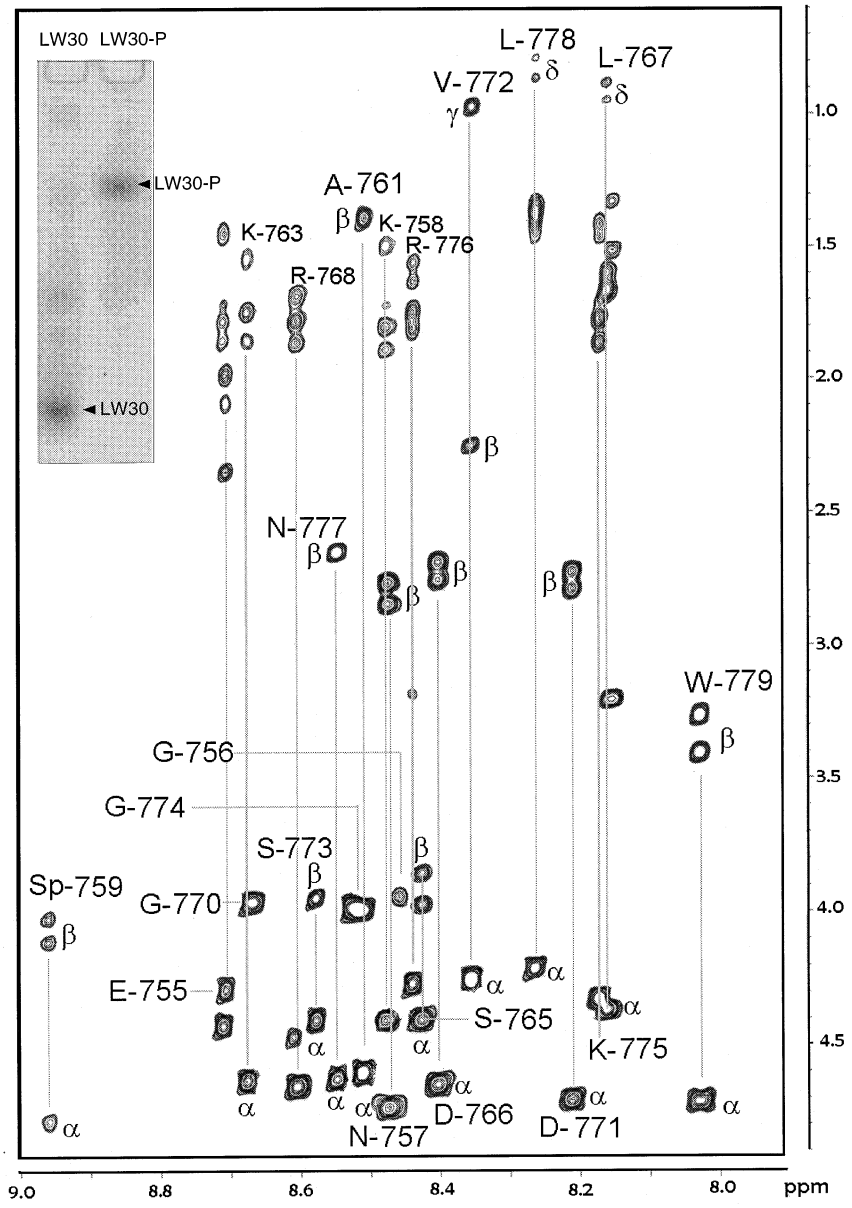


Fig. 1. Amide spectral region of the ^{1}H 2D TOCSY spectrum of LW30-P. The residue-specific cross-peak assignments are shown; conditions: 1 mM, pH 6.4, $T = 285$ K. The resonances of the sole phosphorylated residue, Ser-759, are resolved downfield due to the shift of the amide proton that results from the presence of the phosphate group. The signals of Trp-779 are resolved upfield. (Inset) Phosphorylation of LW30 by MAP kinase resolved on acid urea gel electrophoresis. The stoichiometry and site of phosphorylation were determined using NMR spectroscopy.

ment was achieved by combined use of TOCSY and NOESY cross-peak correlation. Ser-759 was the sole residue of LW30 phosphorylated as seen from the pH sensitivity of the chemical shift of its resonances, readily monitored by correlation with the amide proton signal (Figs. 1 and 4).

3.2. *Phosphorylation of Ser-759 alters the conformation of the turn segment linking the two actin binding sites of LW30*

Although the chemical shift positions of most of the resonances of LW30-P other than Ser-759 were

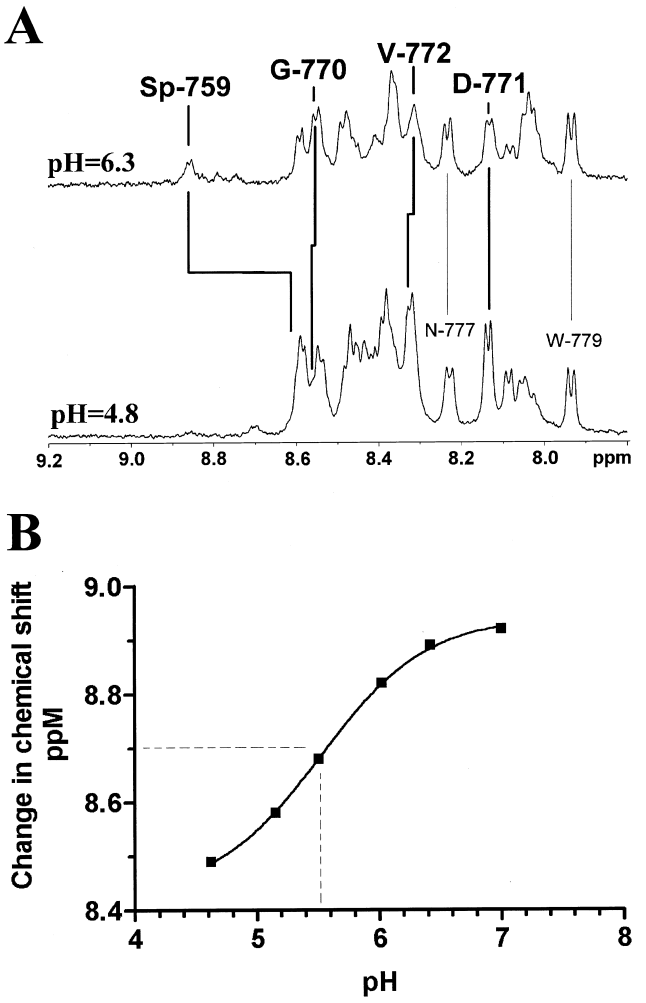


Fig. 2. (A) Amide spectral resonances of LW30-P at pH 4.8 (lower trace) and pH 6.3 (upper trace). The backbone amide resonances that alter chemical shift position upon deprotonation of the phosphate group are labelled in bold type. The large downfield shift of the NH signal of Ser-759 that occurs upon conversion of the monoionic to diionic form of the peptide and the concurrent smaller shifts of the NH signals of Gly-770 and Val-772 are indicated by bold lines. Groups whose amide signals remain unshifted (e.g. Asn-777 and Trp-779) are identified by thin lines. Note also the smaller $^{3}J_{NHC\alpha}$ coupling constant of Asp-771 consistent with a backbone conformational change in the turn segment as a long-range consequence of the ionisation of the phosphate group of Ser-759. (B) The change in the chemical shift of the Ser-759 NH of phosphorylated DS11. The apparent pK_a, 5.5 ± 0.2, obtained by curve fitting is indicated by the dotted line and is the same as that found for LW30-P (data not shown).

closely homologous to those found for the unphosphorylated peptide [22], notable differences were found for the signals of Leu-778 (Fig. 1). The amide proton absorption of Leu-778 (721 in chicken sequence) of LW30-P occurred further downfield ($\Delta\delta \approx -0.1$ ppm) while the α proton and side chain groups of Leu-778 resonated further upfield compared with LW30. These electronic shielding changes indicate a long-range influence of the phosphorylation of Ser-759. Like DS11-P the chemical shift of only a few resonances of LW30-P were found to display changes upon phosphate ionisation. Interestingly, deprotonation of the phosphate group of LW30-P with increasing pH was observed to alter the chemical shifts of the backbone amide signals of some residues in the turn segment of the unphosphorylated peptide, suggesting that the turn conformation of residues 770–773 (713–716, chicken) was sensitive to phosphorylation of Ser-759 (Fig. 2A). NOESY spectra were therefore acquired over a range of pH values across the phosphate pK_a in order to evaluate this in detail.

Inspection of the NOE proximities that characterise the time-average structure of LW30-P in solution showed the occurrence of cross-peaks involving Leu-778 and Trp-779 at the C-terminal end of the peptide (Fig. 4). These proximities were absent from the spectra of the unphosphorylated LW30 peptide (Fig. 4 and [22]) and confirmed the existence of long-range effects due to the phosphorylation of Ser-759. The cross-peaks observed at pH 4.8 (Fig. 4) included the medium range inter-residue connectivities $d_{\alpha N}(i \rightarrow i+2)$ for Gly-770/Val-772 and Asp-771/Ser-773. These proximities define the turn conformation involving residues 770–773 indicating that the turn structure was retained in LW30-P for the mono-ionised form of the phosphate group. We also observed inter-residue NOE between the side chain methyls of Val-772 and the backbone amide protons of Asp-771, Ser-773 and Gly-774, thus providing further confirmatory criteria for the retention of a stable backbone turn in the mono-ionised form of the LW30-P peptide (Fig. 4 and [22]).

Deprotonation of the phosphate group of LW30-P with increasing pH led to the enhancement of the inter-residue proximities at the C-terminus of the peptide indicated by the $d_{\beta N}(i \rightarrow i+2)$ NOE connectivities for Asn-777/Trp-779 (Fig. 4) reflecting a long-range dependence of the backbone conformation on the protonation state of the phosphate group. This long range effect was also apparent for the residues involved in the turn region since the turn-prescriptive

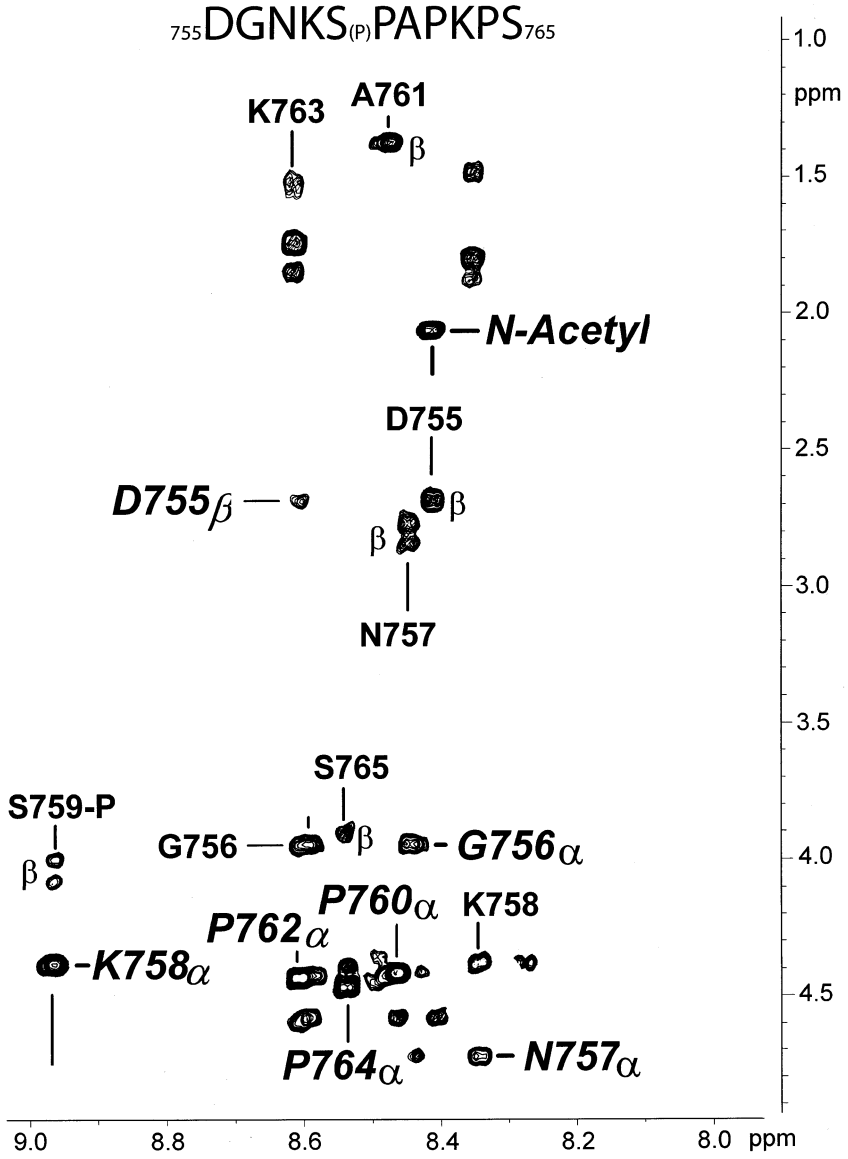
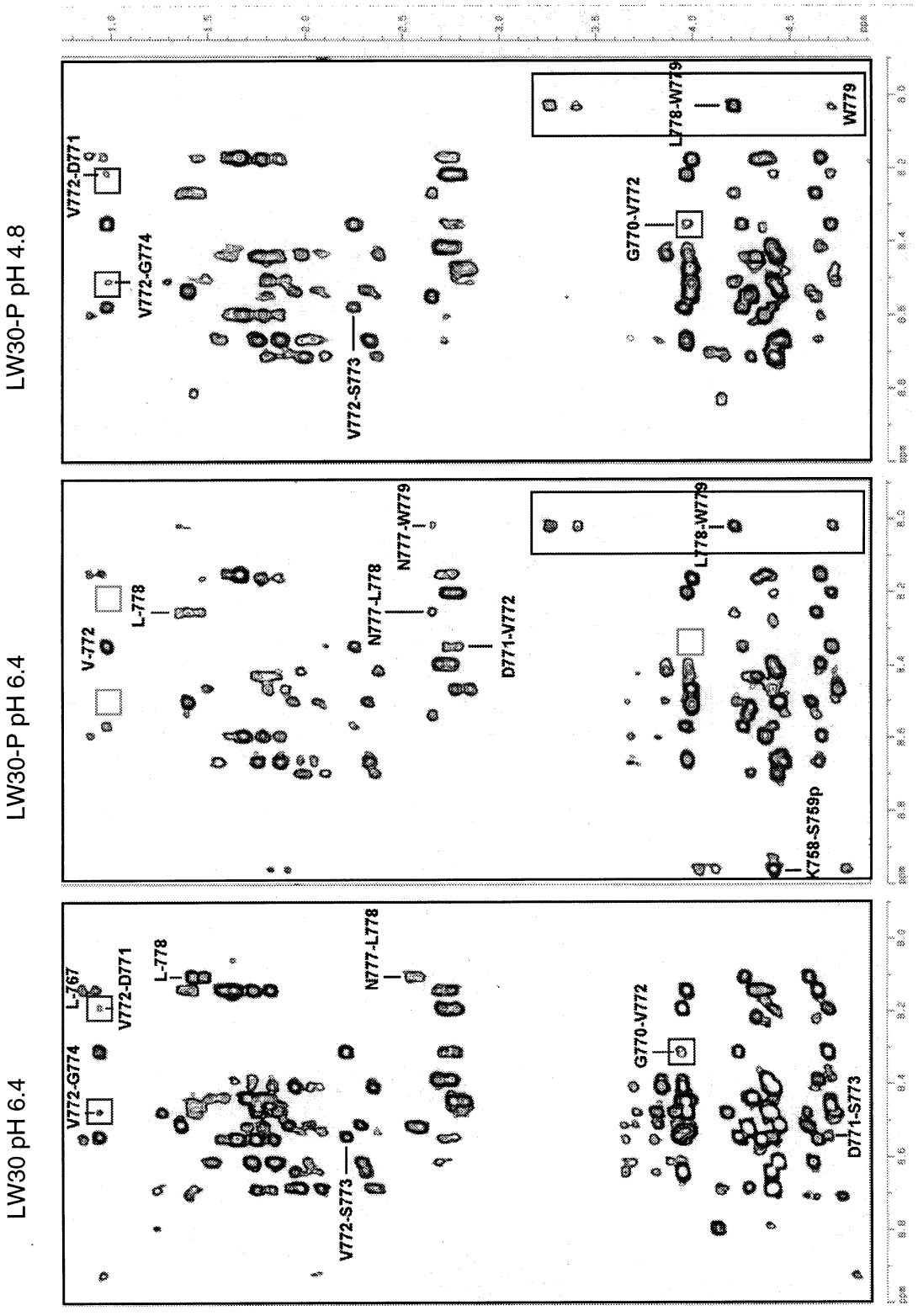


Fig. 3. Backbone NH region of ROESY spectrum of DS11-P. Cross-peaks labelled in bold italics show inter-residue proximities. Cross-peaks identifying intra-residue NOE are labelled in smaller type. Note the $d_{\alpha N}(i \rightarrow i+1)$ correlations for Pro-760, 762 and 764 that together with the corresponding ($X_{H\alpha}$-Pro_{δ}) proximities observed (data not shown) indicate the retention of the predominantly *trans*-X-Pro extended structure in the DS11-P peptide, *N*-acetylDGNKS$_P$PAPKPSamide.

→

Fig. 4. Comparison of the amide spectral region of the ^{1}H 2D NOESY spectrum of LW30-P and LW30. (Left) Unphosphorylated LW30, pH 6.4. (Middle) LW30-P, pH 6.4. (Right) LW30-P, pH 4.8. Identified by the boxed regions are the turn-prescriptive $d_{\alpha N}(i \rightarrow i+2)$ NOE cross-peaks involving residues 770–773 that are readily apparent in the spectra of LW30 and the mono-ionised form of the phosphate group of LW30-P. These cross-peak intensities are notably absent in the di-ionised form of the phosphate of LW30-P (central panel). Backbone to side chain NOE cross-peak intensities in the turn region are also labelled. These are also absent in the di-ionised form of LW30-P whilst new inter-residue cross-peak intensities at the C-terminus are observed. These include the (*i*,*i*+2) correlation of Asn-777 and Trp-779. The remaining cross-peaks do not differ from LW30 [22].


LW30 pH 6.4
LW30-P pH 6.4
LW30-P pH 4.8
V772-G774
L767
V772-D771
L-778
V772-S773
N777-L778
G770-V772
D771-S773
V-772
N777-W779
D771-V772
L778-W779
K758-S759p
V772-D771
W779

$_{750}$L**T**K**T**PDGNKSPAPKPSDLRPGDVSGK**R**NL**W**$_{779}$

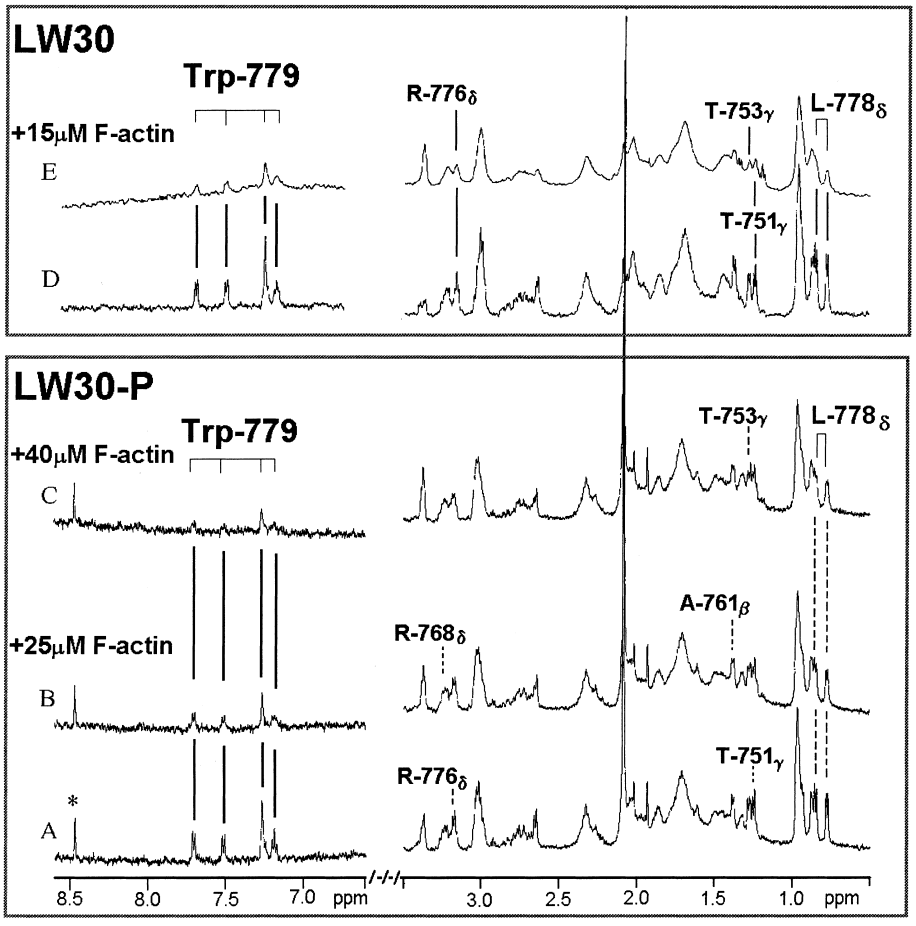


Fig. 5. Proton spectra of LW30-P and LW30 at different stages of titration with F-actin. (Bottom panel) LW30-P. (A) 200 μM LW30-P, pH 7.5, $T = 295$ K. (B,C) 200 μM LW30-P in the presence of 25 μM and 40 μM F-actin respectively. (Top panel) LW30. (D) 200 μM LW30 pH 7.5, $T = 295$ K. (E) 200 μM LW30 in the presence of 15 μM F-actin. The signals markedly affected by interaction with F-actin are labelled and indicated by bold lines. Only the resonances of Trp-779 are notably affected by interaction of LW30-P with F-actin whilst binding of LW30 results in perturbation of resonances at both termini of the peptide, as previously reported [22]. The resonances of groups at the N-terminus of the peptide that remain relatively unaffected by the interaction of LW30-P with F-actin are indicated by dotted lines. The signal marked '*' derives from a buffer impurity. The lack of spectral variation in this resonance upon addition of F-actin indicates that any solution viscosity changes had negligible effects on the spectral resonances. Other resonance assignments are as previously described [22].

cross-peaks for residues 770–773 were absent from the NOESY spectrum of the di-ionised form of the phosphate (Fig. 4). These observations indicated that, while the C-terminus of LW30-P adopted a better-defined conformation as a consequence of the transition to the di-ionised form of the phosphate group, the turn segment linking the two actin binding sites had been destabilised. These distinctive conformational effects were reversible upon alteration of the solution pH.

3.3. Ser-759 phosphorylation of the LW30 peptide results in diminished actin affinity and the loss of dual-sited interaction

The binding of LW30 and LW30-P to F-actin was monitored by 1D ^{1}H-NMR. Different stages of the titration are shown in Fig. 5. Interaction of LW30-P with F-actin is shown by the changes in the resonances of Trp-779 and Leu-778 (C-terminus) with increasing actin concentration. These effects occurred

at much higher mole ratios of F-actin to LW30-P than LW30 (Fig. 5), indicative of diminished affinity upon phosphorylation. For both LW30-P and LW30 signals corresponding to the central residues Thr-751, Thr-753 and Ala-761 remain unaffected by actin. In contrast to the results obtained for LW30 [22], the resonances of groups at the N-terminus of the LW30-P peptide remain unperturbed by interaction with F-actin. These observations indicated that phosphorylation of Ser-759 had led to loss of dual-sited interaction of LW30 with F-actin and that contact was retained only for the C-terminal segment of this minimal inhibitory sequence. This phosphorylation-dependent loss of dual-sited binding to actin was also observed during binding titrations carried out in the presence of tropomyosin (data not shown), indicating that the mode of LW30-P binding was the same when complexed to actin-tropomyosin.

4. Discussion

It has been established that the ability of caldesmon to inhibit actomyosin ATPase activity involves the interaction of non-sequential segments of caldesmon domain 4 with actin [17,20,25–27]. The LW30 peptide, representing the central segment of caldesmon domain 4b, displays inhibition enhanced by tropomyosin and defines the minimum inhibitory sequence [21] whose interaction with actin involves two-pronged docking with tropomyosin strengthening the degree of actin association by both contacts [22]. The residue sequence linking the two actin contacts presumably restricts their spatial orientation and is both necessary and sufficient to impose a distinct geometry on the two actin contact regions.

Phosphorylation at Ser-759 destabilises this geometry and abolishes the ability of caldesmon domain 4b to inhibit actin-activated ATPase. The structural change that alters caldesmon–actin interaction originates from the negative charge of the phosphate group and its potential to participate in extensive H-bond interactions. The NMR studies presented here demonstrate that phosphorylation of Ser-759 (702) in the middle of the spacer sequence linking the two actin contact sites of caldesmon domain 4b resulted in markedly diminished actin affinity and the loss of dual-sited interaction. Only the C-terminal contact with actin (site B′) was retained when Ser-759 was phosphorylated and this correlates with the destabilisation of the bend involving residues 770–773. The conformational influence of Ser-759 phosphorylation is not merely a function of the bulkiness of the covalent modification disrupting a local tertiary structure. The main effects of LW30 phosphorylation are long-range (the turn and site B′ are 12–20 amino acids along the peptide chain) and electrostatic, since the stability of the turn region linking the two actin binding regions was found to be sensitive to the ionisation state of the phosphate group. The charge of the phosphate group here plays an active role in adjusting the local structure to result in greater segmental flexibility of the regions flanking the phosphorylation site. The data reveal a novel mechanism for mediating intermolecular interactions that may be added to the repertoire of conformational control of peptides and proteins by phosphorylation.

This study confirms that two point binding is a minimal and essential requirement for caldesmon inhibition and that the structure of the connecting peptide between actin contact sites B and B′ plays a key role in positioning the actin binding sites so that they can interact with actin in an inhibitory conformation. Phosphorylation within the connecting peptide destabilises this structure and thus appears to be a viable regulatory mechanism consistent with the observed dissociation of caldesmon from thin filaments and reduced inhibition.

Acknowledgements

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