Myc Phosphorylation in its Basic Helix-Loop-Helix Region Destabilizes Transient α-Helical Structures, Disrupting Max and DNA Binding

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ABSTRACT

Myelocystomatosis proto-oncogene transcription factor (Myc) is an intrinsically disordered protein with critical roles in cellular homeostasis and neoplastic transformation. It is tightly regulated in the cell, with Myc phosphorylation playing a major role. In addition to the well-described tandem phosphorylation of Thr-52 and Ser-62 in the Myc transactivation domain linked to its degradation, P21 (RAC1)-activated kinase 2 (PAK2)-mediated phosphorylation of serine and threonine residues in the C-terminal basic helix-loop-helix leucine zipper (bHLH-LZ) region regulates Myc transcriptional activity. Here, we report that PAK2 preferentially phosphorylates Myc twice, at Thr-358 and Ser-373, with only a minor fraction being modified at the previously identified Thr-400 site. For transcriptional activity, Myc binds E-box DNA elements, requiring its heterodimerization with Myc-associated factor X (Max) via the bHLH-LZ regions. Using isothermal calorimetry (ITC), we found that Myc phosphorylation destabilizes this ternary protein–DNA complex by decreasing Myc’s affinity for Max by two orders of magnitude, suggesting a major effect of the phosphorylation on this complex. Phosphomimetic substitutions revealed that Ser-373 dominates the effect on Myc–Max heterodimerization. Moreover, a T400D substitution disrupted Myc’s affinity for Max. ITC, NMR, and CD analyses of several Myc variants suggested that the effect of phosphorylation on the Myc–Max interaction is caused by secondary structure disruption during heterodimerization, rather than by a change in the structurally disordered state of Myc or by phosphorylation-induced electrostatic repulsion in the heterodimer. Our findings provide critical insights into the effects of PAK2-catalyzed phosphorylation of Myc on its interactions with Max and DNA.

Myelocystomatosis proto-oncogene encodes a transcription factor Myc, which is a central regulator of metabolism, proliferation, apoptosis and other key cellular processes (1). Myc is well recognized for its role in maintaining cellular homeostasis and neoplastic transformation. It is deregulated via chromosomal translocations, insertional mutagenesis, and gene amplification, and is the most common gene amplification in human cancers (2, 3). In the absence of binding partners, Myc protein is intrinsically disordered. In particular the bHLH domain (Figure 1A, B) is unstructured, but forms a helical structure when in complex with Max, allowing the heterodimer to interact with E-box DNA
Myc Phosphorylation in the bHLH-LZ Inhibits Binding to Max and DNA and Myc to transactivate or repress genes (4). The transactivation complex is then assembled via recruitment of multiple coactivators to the highly conserved "MYC box" elements in the N-terminal transactivation domain (TAD)(Figure 1B). Because of its oncogenic potential the Myc gene is tightly regulated at transcriptional (5–7), post-transcriptional (8, 9) and post-translational (10) levels.

The close links between Myc and oncogenesis make it an attractive therapeutic target. There have been numerous reports of the identification of small molecule ligands, which bind to the bHLH-LZ domains of Myc and disrupt the interaction with Max (11–14). Compounds working via this mechanism represent the most direct way of modulating the activity of Myc and offer the potential to block Myc action in cancer. However, there have been no reports of such compounds being optimised to demonstrate in vivo efficacy in relevant models. Significant in vivo efficacy targeting Myc has only been achieved through transient expression of an engineered Myc variant termed Omomyc, but detailed biophysical characterisation of the interaction with Myc or Max has not been described (15, 16).

The development of Myc-targeted therapeutics is further complicated by post-translational modifications of Myc, including phosphorylation (17, 18), ubiquitination (19–21), and acetylation (22, 23). Phosphorylation of T58 and S62 in the trans-activation domain has been of particular interest due to their conservation across species and, along with adjacent amino-acids, their location in a hotspot frequently mutated in Burkitt’s lymphoma (24). Interestingly, the phosphorylation of these two residues is interdependent, with the phosphorylation of S62 required prior to T58 (18), which then triggers the proteasomal degradation of Myc (25). Other Myc phosphorylation sites have also been characterized (26–28), including the phosphorylation of T358, S373 and T400 by PAK2(29), which again are highly conserved residues (Figure 1C). T358 is located in the basic region of the bHLH domain, which interacts directly with DNA, whereas S373 and T400 are located in the adjacent helix-loop-helix region, which participates in the interaction with Max (Figure 1A, D). Phosphorylation of these residues has been shown qualitatively to interfere with formation of the Myc:Max:DNA ternary complex and promote the E-box independent regulation of transcription and differentiation (30).

The thermodynamics and kinetics of the Myc:Max:DNA ternary complex formation have been characterized previously, (31–35) but the underlying mechanism for the perturbation of the complex following phosphorylation of residues in the Myc bHLH-LZ region is unknown (29). In this work, ITC, NMR and CD have been used to characterise the effects of these phosphorylation events on the structure of Myc and the affinity of the interaction with Max. The results show that the residual structure in the Myc bHLH-LZ domains in the absence of Max is relatively unperturbed by phosphorylation, but the influence on heterodimer formation is more significant. These observations provide insight to assist in the identification of new therapeutic leads that interfere with the formation of Myc:Max:DNA ternary complex.

RESULTS

Effects of Myc Phosphorylation on Max and DNA binding. — The Myc bHLH-LZ (MycWT) sequence (Figure 1C) is generally well-conserved among species including the three previously identified Ser/Thr residues. These amino acids are all preceded by positively charged residues, although the S373 site does not quite match the (K/R)RX(S/T) consensus sequence of PAK2. In vitro phosphorylation of MycWT by PAK2 results primarily in a dual phosphorylation event, where Myc is predominantly modified at T358 and S373 (MycWT-2P) (Figure 1D, S1-S3, Table S1). This behaviour is apparent the T358 and S373 peaks positions in the NMR spectrum of MycWT-2P in comparison to position of these residues in NMR spectrum of MycWT (Figure 1E-F). Phosphorylation of T358 and S373 peaks positions in the NMR spectrum of MycWT-2P in comparison to position of these residues in NMR spectrum of MycWT (Figure 1E-F). Phosphorylation of T358 and S373 also induces shift of additional resonances belonging to residues close the phosphorylation sites (Figure S4). Phosphorylation of the T400 site is not detectable in the NMR spectra, but mass spectrometry phosphorylation profiling detected low level phosphorylation on this residue and at Y402, S405 and S437 (Table S1 and Figure S1-S3).

The effect of dual phosphorylation of Myc bHLH-LZ on the stability of the Myc:Max:DNA ternary complex was measured using a size exclusion DNA peak shift assay. Compared to MycWT,
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Myc\textsubscript{WT}-2P is impaired in the ability of its heterodimer with Max to bind to E-box DNA (Figure 2A). The Myc\textsubscript{WT}:Max heterodimer binds tightly to E-box DNA, shifting the size exclusion peak to a shorter elution time, whereas the purified Myc\textsubscript{WT}-2P leads to a mixture of free and bound DNA (Figure 2A, S5, S6). The presence of free and bound DNA arise from the fact, that although Myc\textsubscript{WT}-2P has reduced ability to form Myc\textsubscript{WT}-2P:Max heterodimer, Max bHLH-LZ itself can form a dimer and bind to DNA E-Box and induce shift of DNA.

The effect of dual phosphorylation of Myc\textsubscript{WT} on the interaction with Max in the absence of DNA was determined using isothermal titration calorimetry (ITC) (Figure 2B-F, Table 1). Max is known to form a homodimer at high concentration, and dilution of Max into buffer shows an ITC curve that is typical for dimer dissociation (Figure S7), with a dissociation constant $K_D=6\mu$M (Table 1). Therefore, ITC measurements for the Myc:Max interaction were performed with Max as analyte and the Myc variants as the titrants (Figure S8, S9), which minimizes this dilution effect. Under all conditions measured, binding is exothermic, with an unfavourable entropic contribution. In line with previous qualitative studies (29), Myc\textsubscript{WT}-2P has a decreased affinity for Max by 100-fold compared with Myc\textsubscript{WT}. The $K_D$ for binding of Myc\textsubscript{WT} to Max was found to be pH-dependent, with lower pH favouring binding (0.9 nM at pH 6.5 compared with 6 nM at pH 7.4). This effect is maintained on phosphorylation. The differences in binding affinities and in $\Delta H_{bind}$ for Myc\textsubscript{WT} and Myc\textsubscript{WT}-2P binding to Max are the same (within error) at pH 6.5 and 7.4. This implies that an interaction outwith phosphorylation is responsible for the pH dependence, and that the impact of phosphorylation is unrelated to the charge on the phosphate groups. The difference of Gibbs binding energy of Myc:Max heterodimerization upon phosphorylation ($\Delta \Delta G_{phos}$) is 2.5 ± 1 kcal.mol\textsuperscript{-1} at pH 7.4 and 2.7 ± 0.8 kcal.mol\textsuperscript{-1} at pH 6.5.

To provide a broad comparison for the Myc:Max interaction, we also assayed the equivalent interaction between Myc\textsubscript{WT} and Omomyc, which is a Myc derived bHLH-LZ protein that forms a stable homodimer (36) and is reported to interact with Myc \textit{in vivo} (37). However, Myc\textsubscript{WT} binding to Omomyc was too weak to be detected in the ITC experiment (Figure 2G) suggesting that heterodimerization is not the primary effect.

\textbf{Contributions of Individual Phosphorylation Sites.} — Purified Myc\textsubscript{WT}-2P is predominantly phosphorylated on T358 and S373 (Figure 1F). S373 is positioned at the interface between Myc and Max, whereas T358 is distant from this interaction. Its phosphorylation is expected to influence DNA binding only (Figure 1A). Therefore, in order to examine more closely the causes of the decreased affinity of the Myc:Max interaction following phosphorylation, S373 was mutated to Glu and Asp. For comparison, mutation to Glu and Asp of one of the secondary sites of phosphorylation in the heterodimer interface was also carried out. Residue T400 was chosen since this site is highly conserved (Figure 1C), and is the closest of the secondary phosphorylation sites to S373. NMR spectra of the mutant proteins (Myc\textsubscript{S373D}, Myc\textsubscript{T400D}, Myc\textsubscript{S373DT400D}, Myc\textsubscript{S373E T400E}) were compared with Myc\textsubscript{WT}-2P, phosphorylated using PAK2, and showed no significant shifts, indicating that the overall conformation of the protein is maintained (Figure S4). To compare the Asp and Glu mutations more accurately with Myc\textsubscript{WT}-2P, data recorded at pH 6.5 were used as the reference set, as this favours a single negative charge on the phosphate groups, to match the carboxylate sidechains.

In agreement with the behaviour of Myc\textsubscript{WT} and Myc\textsubscript{WT}-2P, ITC measurements at 298K for Myc\textsubscript{S373D} binding to Max gave exothermic binding isotherms with a stoichiometry of 1 (Table 1, Figure 2E, S9). The dissociation constant for Max-binding (23 nM) is comparable to that of Myc\textsubscript{WT}-2P (97 nM), and the small difference (approx. 4-fold) is consistent with a close but slightly imperfect mimicking of a phosphate group by Asp (1). In contrast, the T400D mutation perturbs the interaction much more significantly, altering the stoichiometry to 2:1 Myc\textsubscript{T400D}:Max bHLH-LZ (Figure S9) and increasing the $K_D$ to 270 nM. The combination of these mutations in a S373D/T400D construct would be expected to increase the $K_D$ for Max to approx 6 mM if the effects at the two sites were independent and, consistent with this, no binding was detected in ITC experiments (2F). The effect of mutating to S373 and T400 to Glu was far less pronounced than the equiv-
alent mutations to Asp. Myc373E/T400E binds 9-fold more weakly than MycWT to Max (K_D = 8.2 nM) but 12-fold more strongly than MycWT-2P.

Conformational Perturbation on Phosphorylation. — The perturbation of the Myc:Max interaction by phosphorylation can, in principle, result from a direct disruption of the Myc:Max interface or, since Myc is an intrinsically disordered protein in the absence of Max, through changing the structural propensity of the isolated Myc bHLH-LZ domain. Potential effects of phosphorylation on the structural propensity of Myc were investigated using a combination of NMR and CD spectroscopy. In the NMR spectra of MycWT, MycWT-2P (Figure 1E), all of the Asp variants (S373D/T400D, S373D, and T400D) and MycS373E/T400E (Figure S4), resonances for the C-terminal residues of the bHLH region and the leucine zipper (399-413) are broadened beyond detection so measurements are only available for residues 352 to 398.

One reporter of changes in structural propensity is a change in fluctuations of individual residues, and heteronuclear 15N-1H NOEs are a sensitive reporter of such fluctuations on the ps-ps timescale (38, 39). Most residues in the basic region and helix 1 exhibit intermediate 15N-1H NOE values (≤0.6) (Figure 3A). A fully folded protein of this size would be expected to have NOE values between 0.7 and 0.8, whereas a very dynamic region would be expected to have values between 0.4 and -1.5. The loop and helix 2 are slightly more dynamic than the basic region and helix 1 with 15N-1H NOE values around 0.4. These measurements show that the domain is partially structured and does not behave as a completely disordered protein, though any structure in Myc bHLH-LZ is transient. Phosphorylation of MycWT with PAK2 moderately increases NOE values close to the phosphorylated residues, T358 and S373, indicative of a small increase in local structure. However, this small effect is not reproduced by the substitutions with Asp or Glu residues.

The conformational properties of the Myc variants in the absence of Max was assessed further by deriving secondary structure propensities (40) from 13C_α and 13C_β chemical shifts. Up to 50% helical propensity is estimated in the loop residues and in helix 2 (Figure 3B). Phosphorylation of MycWT has a large effect on residues on the N-terminal side of the phosphorylation sites, where it decreases the α-helical propensity by up to 30% (Figure 3B), in line with other systems (41). Downfield 1H chemical shift changes on phosphorylation indicate that the phosphate group forms a hydrogen bond with its backbone amide, which would compete with hydrogen bonding participating in secondary structure formation.

There is no evidence that phosphorylation leads to a stabilising N-capping effect according to the chemical shift changes of residues to the C-terminal side of the phosphorylation sites. The Asp variants mimicked the chemical shift changes observed on phosphorylation to a large extent, but the Glu variant behaved much more closely to unphosphorylated MycWT (Figure 3B). The decrease in α-helical propensity caused by the S373D mutation is independent of the T400D mutation (Figure 3C), suggesting there is little long range interaction between the sites.

CD spectroscopy was used in conjunction with the NMR measurements to assess the effects of phosphorylation (and mutation) on the secondary structure content of Myc, since NMR approaches were unable to report on the C-terminal region, including residue T400 (42). The Myc:Max heterodimer has approximately 70 ± 9 % α-helical content, with the remainder being random coil (19 ± 5 %) or turn conformations (9 ± 3 %). The isolated Myc variants all have approximately half the per-residue helical content of the heterodimer. All proteins show a Θ minimum at 205 nm resulting from a combination of α-helix, PPII and random coil spectral fingerprints, with the amount of α-helix characterized by Θ at 222 nm (Figure 4A) (42). The differences between the Myc variants are subtle, with only minor changes in the relative values of Θ205 and Θ222.

Full quantification of the differences between the Myc variants using deconvolution of CD spectra into a combination of secondary structure elements (43–45) was not possible, given the data quality below 190 nm and small uncertainties in protein concentrations due to the low ε280 of Myc HLH-LZ. However, using the ratio of Θ222 to Θ205, the α-helical content is ordered WT > S373D >
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S373E/T400E > S373D/T400D > WT-2P > T400D. The α-helical content of the Myc constructs mostly follows the trend seen for the binding affinities for Max, with only MycS373DT400D a significant outlier from this relationship, as it has an undetectable binding affinity using ITC. Ignoring this variant, and expressing the α-helical content as an equilibrium constant relative to the helical content of the Myc:Max complex, gives a correlation coefficient of 0.8, and a slope of 0.08 ± 0.02 for the relationship with log($K_D$) (Figure 4B).

DISCUSSION

Several previous studies have focused on disentangling the kinetics and thermodynamics of Myc:Max dimerization or Myc(Max):Max:DNA ternary complex formation (31–35). In this study, ITC is used to characterize the thermodynamics of Myc and Max bHLH-LZ binding. The measured $K_D$=6 nM at 25°C is substantially lower than the $K_D$=167 nM obtained previously at 23°C by fluorescence anisotropy (32). This difference could be accounted for by the fluorescence labeling of Max (32) or by the use of slightly different constructs (supplementary information). Surprisingly ITC did not reveal an interaction between Myc bHLH-LZ and Omomyc (Figure 2D). Therefore, the cellular effect of Omomyc expression (16, 37) may not be caused through Myc sequestration by Omomyc (46). It appears that the cellular effect of Omomyc rather reflects the competition of Omomyc homodimers with Myc:Max heterodimer for DNA binding sites instead of competition of Omomyc with Max for Myc (or with Myc for Max).

Phosphorylation of Myc bHLH-LZ by PAK2. — The regulation of Myc activity by the action of PAK2 is predicted to be via the disruption of the interaction with E-box DNA, which can occur either directly through perturbation of the DNA binding sites, or indirectly through disrupting the interaction with Max. Identification of the preferred phosphorylation sites can provide information on the relative significance of the two mechanisms. Using a combination of MS and NMR, it is shown that PAK2 phosphorylates Myc bHLH-LZ at multiple sites, but the major phosphorylation sites are at T358 and S373. In dual-phosphorylated Myc (MycWT-2P), T358 and S373 are phosphorylated to the exclusion of all other residues. In the overall phosphorylation mixture, low phosphorylation levels of T400 were detected, and to a lesser extent Y402, S405, and S437, although the phosphorylation of the latter three sites is below the detection level of NMR. This pattern of phosphorylation is broadly in line with previous qualitative observations of Myc phosphorylation by PAK2 (29), but downplays the significance of the phosphorylation of T400.

Dual phosphorylation of Myc significantly affects the Myc:Max heterodimer binding to DNA. The effect of phosphorylation of T358 is readily rationalised from the structure of Myc:Max:DNA ternary complex, since this residue is part of the DNA-binding motif and the phosphate group of pT358 would be juxtaposed with the phosphate diester backbone of the E-box DNA. The effect of phosphorylation of S373 is less clear. Though part of the DNA binding helix, S373 is positioned away from the DNA but adjacent to the Myc:Max dimerisation region, which suggests a perturbation of Myc:Max dimerisation in the reduced stability of the Myc:Max:DNA ternary complex. The sidechain OG atom of S373 is only 3 Å away from the OD1 atom of D74 in the LZ region of Max, and so phosphorylation of S373 has the potential to introduce charge repulsion to this part of the heterodimer interface. Deconvolution of the contributions of the equilibrium between MycWT-2P:Max, Max:Max:DNA, and a potential weaker MycWT-2P:Max:DNA complex is not appropriate for a non-equilibrium technique such as size exclusion chromatography. Consequently, ITC was employed to determine directly the effects of the phosphorylation of S373 on the association of Myc with Max in the absence of DNA.

Compared with MycWT, MycWT-2P has a 100-fold reduced affinity for Max. The S373D mutation reduces the affinity for Max by 20-fold, which potentially leaves a 5-fold (1 kcal.mol$^{-1}$) contribution from the phosphorylation of T358. However, there is significant uncertainty in this value because S373D is not a perfect mimic of pS373. Since T358 is distant from the Myc:Max heterodimer interface in the ternary complex, pT358 would only affect Max binding if there were some "non-native" intramolecular interactions in free Myc, and there is little evidence of long range interactions in any of
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Folded state effects. — Charge repulsion between pS373 of Myc and D74 of Max should have a pH dependence near to the pKa of the phosphate group. The pKa of pSer is usually 5.8-6.2 (41) but this is likely to be increased when pS373 is close to the carboxylate group of D74 of Max. As a result, the interaction between MycWT-2P and Max is expected to be affected significantly between pH 6.5 and pH 7.4. There is an effect of pH on the Myc:Max binding thermodynamics in this pH range but it occurs independently of the phosphorylation of S373. Hence, the observed effect is most likely due to H81 of Max, the imidazole NH groups of which are hydrogen bonded by E410 and E417 of Myc in the heterodimer, which would require protonation of H81 at pH 7.4. The lack of a phosphorylation-dependent pH effect is further unexpected, because the specificity of leucine zipper interactions is dictated by the charged residues which flank the core hydrophobic interface (47) and the electrostatic environment of Myc is modified by the introduction of anionic phosphate groups. Consequently, the results suggest that repulsion between pS373 of Myc and D74 of Max is not a main contributory factor to the reduction of affinity for Max caused by the dual phosphorylation of Myc. This conclusion is supported by the negligible effect that the MycS373D/T400D variant has on the Myc:Max interaction, even though the Glu carboxylate oxygen atoms and the phosphate oxygens in pSer and pThr are separated by the same number of bonds from the backbone Cα.

Conformational ensemble effects — The effects of dual phosphorylation are not limited to electrostatics, but can also include the disruption of all equilibria that affect the overall thermodynamics. In order to simply describe the binding of Myc to Max, a 4-state mechanism, which separates the coupled binding and folding into two events, was used as a thermodynamic model (Figure 4C; note, our measurements do not report on the pathway for this interaction). In the absence of Max, an ensemble of partially disordered Myc conformers is in rapid equilibrium with fully disordered Myc (equilibrium 1), which is the equilibrium observed using NMR. Rarely (and thus invisible to NMR), the partially disordered ensemble will populate a fully folded, monomeric Myc (equilibrium 2). Association with Max allows stabilisation of the fully folded Myc in the heterodimer (equilibrium 3). The arguments above concentrate on equilibrium 3.

Some effects of dual phosphorylation on equilibrium 1 are visible in the NMR-derived parameters. Normally, helical regions which are C-terminal of phosphorylation sites are stabilised through N-capping interactions, while helical regions N-terminal of phosphorylation sites are destabilised by disruption of the helix hydrogen bonding (41). Phosphorylation of S373 leads to no measurable helicity at residue 373 and has a substantial destabilising effect on both the N-terminal and C-terminal side. The S373D and S373D/T400D variants behave similarly to each other, but the destabilising effects are focused on the N-terminal side of the mutation and are significantly less than for pS373. Phosphorylation of T358 leads to a response in its vicinity that is more reminiscent of the S373D mutation than S373 phosphorylation. Helix propensity is lost completely on its N-terminal side (where it was already low) but is moderately unaffected at position 358, and on its C-terminal side, where the bulk of helicity associated with equilibrium 1 resides. Hence, the primary effect of phosphorylation on equilibrium 1 appears to result from local secondary structure perturbation by pS373. The similarity in behaviour between the effects of pS373 and S373D mutations, but not S373E mutations, strongly implicates the competition between helix formation and sidechain-backbone hydrogen bonding to be the most significant factor in the perturbation of equilibrium 1.

Overall, the changes in helical propensities reported in the NMR data are small, which is consistent with the modest changes in CD spectra between the same species. Intriguingly though, there is a good correlation (Figure 4B) between the perturbation of secondary structure formation in the monomer and the free energy of the Myc:Max binding interaction across the phosphorylated and mutated Myc species. However, this correlation accounts for less than 10% of the observed effects on binding. This leaves a strong implication that the perturbation of secondary structure formation in the monomer by phosphorylation is a reflection of a more major effect on equilibrium 2. This would mean that the ensemble of partially disordered Myc
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Conformers formed in equilibrium 1 is more capable of accommodating the perturbation introduced by phosphorylation (as, for example, reported by the NMR data in the vicinity of pS373) than the more structured, extended helical conformation that is required in order to bind to Max in the wild-type mode (equilibrium 3).

Comparison with pT400 — T400 is a canonical PAK2 phosphorylation site, even though the pT400 modification is not prevalent in the purified MycWT-2P samples. Notably, the effects of introducing a charged group here are more significant than at S373. T400 is located near the C-terminus of helix 2, which is adjacent to helix 1 of Max in the ternary complex. The T400D mutation has the largest effect on Max binding, and on the conformational ensemble of unbound Myc (as detected by CD spectroscopy). Surprisingly though, the Myc7400D-Max complex switches to become heterotrimeric. Whilst such leucine zipper complexes have been reported previously and the rules governing homotrimeric structure are well understood (48), it is not clear how this single point mutation alters the preference over dimer in this complex. The line broadening observed in the C-terminal region of the Myc bHLH region prevents a full comparison of the NMR and CD data, but it is apparent from the fact that the perturbations introduced by the T400D mutation are not widespread in equilibrium 1.

In summary, Myc is an intrinsically disordered protein (IDP) which elicits its biological effect by adopting a more ordered structure that enables it to interact with Max and affect transcription at the DNA level. Post-translational modifications of IDPs are key mechanisms for regulating this class of proteins. The data presented here provides an insight into the structural effect of PAK2 catalysed phosphorylation of Myc. Further characterisation of phosphorylated Myc could provide information to assist in the design of small molecule ligands targeting this state. Stabilisation of the phosphorylated form could be a mechanism for disruption of Myc function in cancer. Identification of suitable ligands targeting this mechanism remains a challenge.

EXPERIMENTAL PROCEDURES

All protein constructs and mutants (Supplementary Information) were generated by gene synthesis with an N-terminal hexahistidine tag and tobacco etch virus (TEV) protease site. All were subcloned into a pET-9 vector for Escherichia coli expression. Protein expression was performed in BL21-Gold (DE3) (Novagen) E. coli cells induced using 0.5 mM IPTG for 4 hours at 37°C.

Cells were resuspended in PBS buffer containing Complete Protease Inhibitor tablets (Roche) and benzonase nuclease (2.5 u/ml) were lysed using a Constant Systems cell disruptor at 25 k.s.i. Guanidium chloride was added to resuspended cells to 4 M final concentration and stirred for 1 h. Guanidium containing lysate was further clarified by centrifugation at 75000g for 1 h.

Proteins were purified from supernatant using nickel affinity chromatography followed by Superdex 75 gel filtration chromatography (50 mM Hepes pH 7.5, 500 mM NaCl, 1 mM EDTA and 1 mM DTT) and the His tag was cleaved by TEV protease. Cleaved protein was separated in denaturing conditions (4 M guanidium chloride and 2 mM CaCl2 to chelate the EDTA) as flow through on nickel chromatography. Protein was concentrated by ultra-filtration (3 kDa pore) and purified and buffer exchanged on Superdex 75 gel filtration chromatography (50 mM potassium phosphate pH 6.5, 500 mM ammonium chloride, 1 mM EDTA, 1 mM DTT).

Phosphorylation by PAK2 T402E (Dundee University) was carried out at room temperature in a mixture of Myc 0.5 mg/mL, PAK2 3 µg/mL, and ATP 300 µM, each in phosphorylation buffer (60 mM HEPES pH 7.5, 3 mM MgCl2, 3 mM MnCl2, and 1.2 mM DTT). The phosphorylation was carried out until the level of dual phosphorylation was higher than 80% as quality controlled by mass spectrometry (C18 reverse phase LC-ESI-Q-TOF). The reaction was quality controlled each 24 h and if the level of double-phosphorylation was unsatisfactory additional 3 µg/mL of PAK2 and 0.3 µMol/mL ATP was added.

Dual-phosphorylated Myc was further purified on cation exchange. ResourceS 1.0 mL columns were equilibrated with 60 mM HEPES pH 7.5 and eluted over 20 CV with 60 mM HEPES pH 7.5, 1M NaCl. The fractions containing the dual-phosphorylated Myc were identified by the mass spectrometry (Figure S6), pooled and buffer exchanged to 50 mM potassium phosphate pH 6.5,
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500 mM ammonium chloride, 1 mM EDTA, 1 mM DTT.

Gel filtration mobility shift assays were performed using a Superdex 75 3.2/300 column equilibrated in 50 mM potassium phosphate pH 6.5, 500 mM ammonium chloride, 1 mM EDTA, 1 mM DTT using 5 µL injections of sample comprising 10 µM E-Box DNA alone or in combination with 10 µM Myc and/or 10 µM Max. The detector was set to λ=260 nm to detect DNA. E-Box DNA was prepared by annealing of chemically synthesized oligonucleotides (supplementary information).

All ITC experiments were performed on a VP-ITC instrument at 25°C. In all titration experiments the 20 µM, 40 µM, and 80 µM Myc bHLH-LZ was titrated into Max bHLH-LZ at 2 µM, 4 µM, and 8 µM concentrations. The concentrations of Myc and Max bHLH-LZ were initially determined by amino acid analysis and interpolated to a Bradford assay. All experiments were performed in 50 mM potassium phosphate pH 6.5 (or pH 7.4), 500 mM ammonium chloride, 1 mM EDTA, 1 mM DTT. Max bHLH-LZ dissociation titration was obtained by titrating 160 µM Max bHLH-LZ into buffer. All experiments were analyzed using NITPIC (49) and SEDPHAT/ITCsy (50, 51). The ITC titration parameters were obtained from simultaneous fit of titrations at 20 µM, 40 µM, and if available 80 µM concentrations of Myc bHLH-LZ.

All NMR experiments were performed at 4°C. All heteronuclear 15N-{1H}NOE spectra and assignment spectra of MycWT and MycWT-2P were acquired on Bruker Avance III 800 MHz, while assignment spectra of MycS373E/T400E, MycS373D/T400D, MycS373D, and MycT400D were acquired using a Bruker Avance III 600 MHz. Both spectrometers were equipped with a 5-mm z-gradient 1H/13C/15N TCI probe. The heteronuclear 15N-{1H}NOE experiments were acquired using hsqnoef3gpsi pulse sequence with 7 s relaxation delay. The BEST type HNCA, HN(CO)CA, HNCACB, and HN(CO)CACB experiments (52, 53) were used for assignment. Secondary structure propensities were obtained by SSP (40) using Cα and Cβ only, with internal SSP chemical shift referencing.

For CD experiments, all proteins were buffer exchanged into 50 mM potassium phosphate, pH 6.5 (or pH 7.4) 500 mM potassium fluoride. CD spectra were recorded at 293 K in a Jasco J-810 spectrometer equipped with a Peltier temperature controller in a 0.1 cm path length quartz cuvette, containing protein at 0.3 mg/mL. Blank spectra were recorded with the same buffer in the absence of protein and subtracted from the protein spectra. All measurements for CD spectra were taken in triplicate with a response of 2s, 0.1 nm data pitch, 1 nm band width and scanning speed of 20 nm/s from 260 - 185 nm. To obtain secondary structure composition, data were analyzed using Contin-LL method (44) using reference set number 7 (54).

The mass spectrometry analysis of the MycWT-2P phosphorylation pattern was performed on SDS PAGE with gel bands excised from a Coomassie Stained gel and subjected to reduction, alkylation and digestion with trypsin (55). For ESI MSMS using the Qstar Elite (ABSciex) mass spectrometer, 5ul of the sample was chromatographed using the U3000 Ultimate (Thermo) nanoflow chromatography system and the outlet flow run directly into the Qstar Elite for analysis via the nanoflow probe at a flow rate of 300nl/min. A 20 minute reversed phase gradient was run using a 300um i.d. x 5mm C18 PepMap trapping pre-column and a 75um i.d. x 15cm C18 PepMap analytical column. The Qstar collected data in positive ion mode and an autoswitching setup was initiated with automatic precursor selection based on peak intensity and charge state. The collision energies were automatically adjusted based on the precursor. Nitrogen was used as the collision gas. The subsequent data files generated were searched against in the Myc sequences using the Mascot Daemon software. The searches were then manually verified.

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Conflict of interest: The work described here was initiated as a project within AstraZeneca. KJE, RAD,
GAH and JWMN are current employees of AstraZeneca and may hold stock in the company. PM is a former employee of AstraZeneca and may hold stock in the company. JPW and MJC are employees of The University of Manchester.

**Author contributions:** P.M. designed experiments, designed, expressed and purified protein constructs, carried out NMR experiments, interpreted data and wrote manuscript. K.J.E. designed and carried out NMR experiments. G.A.H. designed and carried out CD measurements and participated in analysis and interpretation of biophysical data. S.P. carried out and analyzed NMR experiments. M.J.C. designed, carried out and analyzed NMR experiments, analyzed CD data and wrote the manuscript. W.J.M.N. designed and coordinated experiments. J.P.W. designed and coordinated experiments. R.A.D. designed and coordinated experiments. All authors discussed results and commented on the manuscript.
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FOOTNOTES
Footnotes should include details such as grant information, and any abbreviations used within the article.

TABLES

|            | $K_D$[nM] | $\Delta G$[kcal.mol$^{-1}$] | $\Delta H$[kcal.mol$^{-1}$] | $\Delta S$[kcal.mol$^{-1}$] |
|------------|-----------|-----------------------------|----------------------------|-----------------------------|
| pH 7.4     |           |                             |                            |                             |
| MycWT      | 6±1       | -11.2±0.1                   | -18.2±0.4                  | 7.0±0.6                     |
| Myc$_{WT-2P}$ | 376±7   | -8.77±0.01                  | -14.08±0.01                | 5.31±0.02                   |
| pH 6.5     |           |                             |                            |                             |
| MycWT      | 0.9±0.5   | -12.4±0.4                   | -25.6±0.6                  | 13.3±1.0                    |
| Myc$_{WT-2P}$ | 96.9±0.1 | -9.6±0.4                    | -20.2±0.1                  | 10.6±0.5                    |
| Myc$_{T373E/T400E}$ | 8.0±1.0 | -11.0±0.1                   | -24.0±2.0                  | 13.0±2.0                    |
| Myc$_{T373D/T400D}$ | n/a    | n/a                         | n/a                        | n/a                         |
| Myc$_{S373D}$ | 23.0±9.0 | -10.4±0.2                   | -22.±1.0                   | 12.0±1.0                    |
| Myc$_{T400D}$ | 270.0±50.0 | -9.0±0.1                   | -21.0±2.0                  | 12.0±2.0                    |
| Max diss.  | 5600±600  | -7.2±0.1                    | 6000±2000                  | 6000±2000                   |

Table 1: ITC derived dissociation constants and thermodynamic parameters of Myc bHLH-LZ variant upon interaction with Max bHLH-LZ. * stoichiometry for this reaction is 0.5
FIGURES
Figure 1: Myc structure and phosphorylation. A. Crystal structure of the heterodimer formed by the bHLH-LZ domains of Myc (blue) and Max (orange), in complex with E-box DNA (pdb: 1NKP), showing potential phosphorylation sites as red space-filling spheres. B. Domain structure of Myc protein (Mb - Myc box). C. A sequence logo representing Myc bHLH-LZ based on a Hidden Markov Model. Higher stacks represent increased conservation of a residue. D. Secondary structure of Max bound Myc including the phosphorylation sites detected in this study. Relative phosphorylation of different sites is qualitatively represented with red filling. E. \(^1\)H-\(^{15}\)N HSQC spectrum fingerprint of unphosphorylated (black, ID: 27414) and phosphorylated (green, BMRB ID: 27422) Myc bHLH-LZ (fully assigned in Figure S4) with marked phosphorylation sites. F. 1D slices (in \(^{15}\)N) of \(^1\)H\(^{15}\)N HSQC peaks for T358, S373 and T400 in unmodified (black) Myc and Myc doubly phosphorylated by PAK2 (blue).
Figure 2: A. Size exclusion profiles of E-box DNA in the absence (red) and presence of Max and unmodified (blue) and PAK2 phosphorylated (green) Myc WT. B,C. ITC titration curves of unphosphorylated (B) and phosphorylated (C) Myc WT binding to Max at pH 7.4. D,E. ITC derived thermodynamic parameters of Myc bHLH-LZ variants binding to Max at pH 7.4 (D) and pH 6.5 (E). F,G. Flat profiles of ITC integrated heat data of 8µM Myc S373D/T400D titration to 80µM Max (F), and 8µM Myc WT titration to 80µM Omomyc (G) revealing no interaction between the proteins in vitro. All ITC experiments were performed with Myc WT, Myc WT-2P, Myc mutant, and OMOMYC as titrants and Max as analyte.
Figure 3: NMR derived ps-ns timescale dynamics (A) and secondary structures (B, C) of Myc bHLH and its variants including the schematics of Myc bHLH-LZ secondary structure. (A) Heteronuclear $^{15}$N-$^1$H NOE of Myc bHLH-LZ and its variants. pT358 and pS373 phosphorylation sites are marked with an arrow. (B, C) Secondary structure propensities of Myc bHLH and variants obtained from $C^\alpha$ and $C^\beta$ chemical shifts.
Figure 4: Phosphorylation induces destabilization of the α-helical structure of Myc bHLH-LZ. (A) Circular dichroism spectra of Myc variants (blue), compared with that of the Myc:Max complex (black). (B) Relation between $K_D$ and relative helix content ($\Theta_{205}/\Theta_{222}$ ratio) and disordered structures obtained from circular dichroism. (C) 4-state model for folding and binding of Myc:Max heterodimer. Phosphorylation of Myc$_{WT}$ by PAK2 destabilizes α-helical structures of Myc bHLH-LZ (wide to narrow ribbon transition). Decreased sampling of α-helical structures participate to the shift of the Myc:Max equilibrium towards the free Myc bHLH-LZ. Myc bHLH-LZ to Max bHLH-LZ binding stabilizes the α-helical structures of both, Myc and Max.
Myc Phosphorylation in its Basic Helix-Loop-Helix Region Destabilizes Transient α-Helical Structures, Disrupting Max and DNA Binding
Pavel Macek, Matthew J Cliff, Kevin J. Embrey, Geoffrey A. Holdgate, J. Willem M. Nissink, Stanislava Panova, Jonathan P. Waltho and Rick A. Davies

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