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A moving target: structure and disorder in pursuit of Myc inhibitors

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Abstract

The Myc proteins comprise a family of ubiquitous regulators of gene expression implicated in over half of all human cancers. They interact with a large number of other proteins, such as transcription factors, chromatin-modifying enzymes and kinases. Remarkably few of these interactions have been characterized structurally. This is at least in part due to the intrinsically disordered nature of Myc proteins, which adopt a defined conformation only in the presence of binding partners. Due to this behaviour, crystallographic studies on Myc proteins have been limited to short fragments in complex with other proteins. Most recently, we determined the crystal structure of Aurora-A kinase domain bound to a 28 amino acid fragment of the N-Myc transactivation domain. The structure reveals an α-helical segment within N-Myc capped by two tryptophan residues that recognize the surface of Aurora-A. The kinase domain acts as a molecular scaffold, independently of its catalytic activity, upon which this region of N-Myc becomes ordered. The binding site for N-Myc on Aurora-A is disrupted by certain ATP-competitive inhibitors, such as MLN8237 (alisertib) and CD532 and explains how these kinase inhibitors are able to disrupt the protein-protein interaction to effect Myc destabilization. Structural studies on this and other Myc complexes will lead to the design of protein-protein interaction inhibitors as chemical tools to dissect the complex pathways of Myc regulation and function, which may be developed into Myc inhibitors for the treatment of cancer.

Introduction

Myc proteins are transcription factors that markedly alter gene expression through both activation and repression [1-3]. The three Myc protein family members in humans (c-Myc, N-Myc, L-Myc) are all aberrantly expressed in cancers. Myc overexpression dramatically changes the landscape of gene expression, altering many of the cellular properties that are hallmarks of cancer e.g. resistance to cell death, sustained proliferation and promotion of angiogenesis [4]. Inhibition of Myc is a validated therapeutic strategy, but the development of clinical compounds that target intrinsically disordered proteins (IDPs) such as Myc directly is a daunting challenge due to the absence of stable and well-defined pockets that could be probed with small molecules [5,6]. Because of these difficulties, many attempts have been made to inhibit Myc indirectly, through inhibition of targets that act downstream of Myc in various Myc-driven cellular processes [7] or upstream of Myc, such as inhibition of BRD4 recruitment to chromatin using BET bromodomain inhibitors, which is effective in many Myc-driven cancers because BRD4 is often required to drive transcription of *MYC* itself [8]. Here we review the opportunities for targeting the Myc proteins and complexes in which

small regions of Myc become ordered through binding to partner proteins. Finally, we discuss a recently postulated strategy for targeting the stability of Myc through one such binding partner, Aurora-A.

Structural basis of Myc interactions

While Myc polypeptides alone are intrinsically disordered, and are likely to exist in multiple conformation states simultaneously, there are transient secondary structure elements that, in some cases, become conformationally stable while in complex with a binding partner [9]. In contrast to the emergent methods that might be used to develop small molecule ligands that recognise disordered polypeptides, the ordered regions glimpsed in structures of Myc complexes could be targeted using established structure-based drug discovery approaches that promise greater target specificity. However, there are currently only four crystal structures of Myc complexes: Importin-α [10], Max [11], WDR5 [12] and Aurora-A [13] (Figure 1). In many of these structures, the Myc fragment forms a short, extended peptide sequence and only in the complexes with Aurora-A and Max does Myc form intrachain hydrogen bonds. c-Myc, N-Myc and L-Myc have regions of sequence homology that mediate interactions with critical partner proteins (Figure 1) [14]. The most C-terminal of these regions forms an essential DNA-binding domain through formation of a basic helix-loop-helix leucine zipper (bHLHZ) domain in complex with Max [10]. Other conserved sequence motifs called Myc boxes (MB0-IV,) serve as docking sites for protein-protein interactions. The Myc transactivation domain (TAD), spans the N-terminal conserved motifs MB0, MBI and MBII.

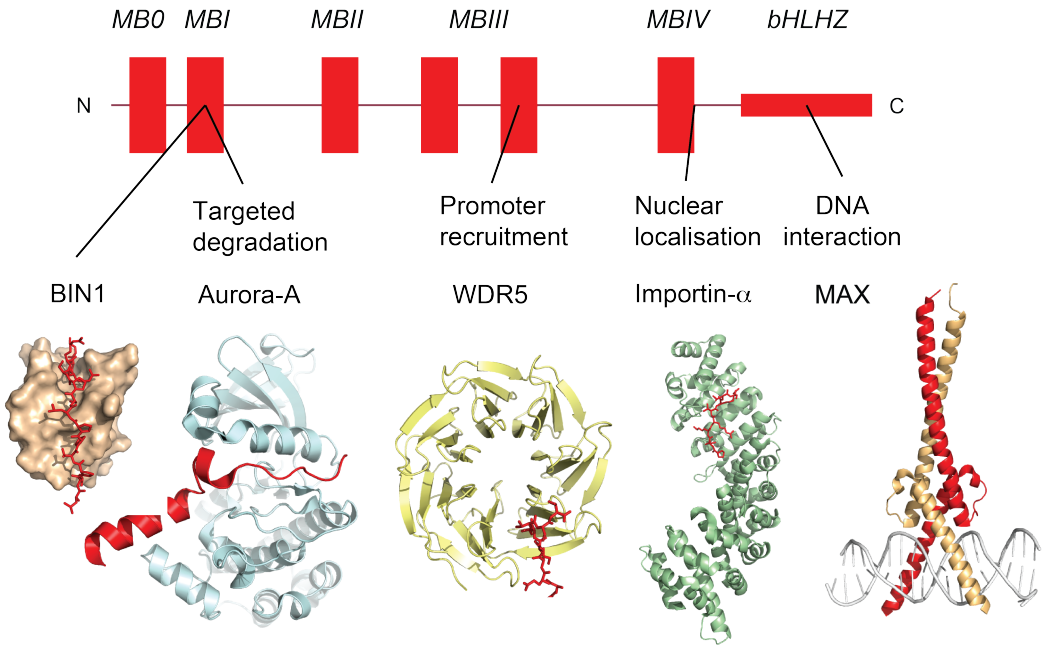

Figure 1. Overview of Myc structures. From the top: domain structure of Myc proteins showing conserved regions as red boxes; MB, Myc box; bHLHZ, basic helix-loop-helix leucine zipper; functional annotation of regions and the proteins that interact at these sites; structures of protein complexes containing Myc (red). The PDB entries are, from left to right, 1MV0, 5G1X, 4Y7R, 1EE4, 1NKP).

The crystal structure of the bHLHZ Myc-Max complex bound to double-stranded DNA shows that an extensive, mainly hydrophobic, buried interface is formed between the Myc and Max

leucine-zipper regions with register and heterodimer specificity apparently maintained by two pairs of polar residues (Arg423/Arg424 of Myc and Gln91/Asn92 of Max) which form a small interfacial hydrogen bond network [10]. The bHLHZ region of the Myc-Max heterodimer interacts directly with the major groove of DNA most frequently in gene promoter regions containing E-boxes of sequence 'CACGTG'. Myc and Max each recognise half of the palindromic sequence through a set of similar, sequence-specific protein-DNA interactions [10]. Nuclear magnetic resonance (NMR) assignments are also available for the bHLHZ domains of c-Myc and v-Myc [15-18]. Co-solution structures have been determined of the c-Myc leucine zipper and a full bHLHZ Myc-Max complex in the absence of DNA [17, 18] which both resemble the co-crystal structure of the DNA-bound Myc-Max complex [10]. Inhibition of the Myc-Max interaction has been achieved using a version of the Myc bHLHZ domain containing four mutations within the leucine zipper that allow it to form relatively inactive dimers with both Myc and Max [19, 20]. The recruitment of Myc-Max to sites on chromatin depends on other chromatin-associated factors such as the β-propeller protein WDR5, which binds Myc through a highly conserved acidic/hydrophobic sequence within the MBIIIb motif [12]. The complementary basic/hydrophobic binding groove lies on one face of the WDR5 β-propeller and also mediates interactions with RBBP5 and KANSL2 via sequence motifs similar to the one found in MBIIIb. Since WDR5 also recognises, via other binding sites, various histone modifications that are characteristic of active chromatin it is able to influence the selection of Myc target genes through recruitment to sites rich in such marks.

NMR spectroscopy is well-suited to study the interactions of intrinsically-disordered proteins. For example, backbone resonance assignment has been performed for the full length murine B-Myc [21], a rodent Myc homologue which contains only the TAD but shares approximately 65% amino acid identity with the c-Myc TAD. Using this data, interactions of Prefoldin subunit 5 (PFDN5) and TATA-binding protein were demonstrated with sequences within and flanking B-Myc MBI and MBII. NMR has also been used to investigate how the interaction between c-Myc and Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1) is regulated by Cdk1 phosphorylation [9]. c-Myc interacts with PIN1 via MB0 in the absence of phosphorylation while interaction via MB1 requires phosphorylation at Ser62. Chemical shift perturbations were also used to map the binding site of c-Myc on PIN1: chemical shifts induced by binding of unphosphorylated c-Myc were restricted to a cleft between the WW and PPIase domains while binding of c-Myc phosphorylated at Ser62 affected a much larger proportion of resonances spread throughout the entire protein leading the authors to propose that c-Myc and PIN1 form a fuzzy multivalent complex which is anchored by the primary point of contact between c-Myc MB0 and the interdomain cleft of PIN1 [9]. A solution structure was determined of residues 55-66 of c-Myc in complex with the C-terminal SH3 domain of BIN1. In this structure, Ser62 of c-Myc is positioned close to an acidic patch on the binding pocket surface suggesting a mechanism by which phosphorylation of this site negatively regulates this interaction [22]. More recently, BIN1 was observed to interact with with several other sites in the TAD suggesting that this may be another example of a Myc forming a multivalent complex [23].

Myc interacts with many other binding partners, a summary of which has been provided in a recent, comprehensive review [14]. These binding partners are involved in transcriptional activation (e.g. histone acetyltransferases, P-TEFb), transcriptional repression (e.g. Miz-1) and the regulation of Myc protein turnover. The development of inhibitors that block the interaction of Myc with chromatin, and its subsequent recruitment of the transcriptional regulatory apparatus, is a major avenue of current investigation [12, 19, 20].

Targeting Myc as an IDP

Both the N-terminal regulatory region and the C-terminal bHLHZ domain of Myc are intrinsically disordered in the absence of binding partners, as reported by circular dichroism (CD) and NMR spectroscopies [9], but as described above, regions of the protein do adopt ordered conformations upon binding to interaction partners. In contrast to the spontaneous transition of a globular protein to its folded state driven by the physicochemical properties of the polypeptide, an IDP merely carries the potential to adopt a particular conformation, or samples that conformation among a diverse conformational ensemble, but requires its binding partner to act as a template for folding and stabilisation of a single ordered state [24]. The transition of a region of the c-Myb TAD to an α-helical conformation upon binding to the CREB-binding protein was investigated as an example of "templated folding" mode of IDP interaction [24]. However, as mentioned above, Myc may interact with some of its binding partners transiently across multiple binding sites: in this multivalent mode of binding, which is reportedly common among IDPs, at any moment across the population of the IDP molecule, each individual binding motif may be partly bound/ordered and partly unbound/disordered [9, 23, 25].

Small-molecule inhibitors that bind to IDPs and disfavour their transition to an ordered conformation competent to form a stable interaction are an emergent concept and there are no drugs in clinical use that are known to work in this way. However, targeting the c-Myc bHLHZ domain by these means has seen some early success. Myc bHLHZ and Max form their ordered DNA-binding structure only upon dimerisation and as monomers they are both disordered. Many groups who have screened compound libraries for small molecules able to inhibit the Myc-Max interaction have discovered compounds that recognise and stabilise the disordered monomeric forms of Myc and Max, disfavouring formation of the ordered heterodimer [26]. Ligands such as the benzylidene rhodanine compound 10058-F4 were shown to bind to multiple sites within the c-Myc bHLHZ domain causing dissociation of Myc-Max complexes and transition from an α-helical to a disordered state as observed by CD and NMR spectroscopies [27-29]. Molecular dynamics simulations of 10058-F4 interactions with c-Myc bHLHZ domain peptides suggested that the compound binds weakly to a wide range of peptide conformational states through hydrophobic interactions but with no dominant binding mode [30]. It has been suggested that the enhanced conformational freedom of the c-Myc polypeptide in the presence of the compound results from inhibition of intramolecular interactions and that this increases the affinity of the compound for the protein, and energetically favours its disordered state, through increased entropic contributions to the system [31]. While target specificity is a concern with this mode of action and with the rhodanine scaffold [32], some selectivity for Myc-Max dimers over other transcription factors was demonstrated for 10058-F4 [27] and optimisation of IDP-binding compounds for Myc-Max selectivity has been reported [26, 33].

The regulation of Myc stability

Myc proteins are turned over rapidly, with a short half-life of ~20 minutes in non-transformed cells and many cancer cells [34]. Since they are powerful oncoproteins, tight post-translational control of Myc protein levels is clearly crucial. Furthermore, recent work has suggested that the turnover of Myc protein is a necessary stage in Myc-driven transcription [35]. The degradation of Myc is mediated by the ubiquitin-proteasome system, a multi-protein cascade that conjugates ubiquitin through Lys48-linked chains attached to

Lys residues on substrate proteins [36, 37]. Myc proteins are modified by several different E2/E3 pairs, whose activities are antagonized by deubiquitylating enzymes (DUBs) [38, 39].

One of the critical pathways that governs Myc stability involves the E3 SCF^{Fbxw7} and E2 cdc34 acting on a phosphorylated form of Myc that is generated as a consequence of a series of post-translational modifications [39]. Myc is first phosphorylated on Ser62 by Cdk1/cyclin B, which together with proline isomerization catalyzed by Pin1, increases the transcriptional activity of Myc. Phosphorylation of Ser62 also primes Myc for phosphorylation at Thr58 by Gsk3β. A combination of a second Pin1-mediated proline isomerization event, and dephosphorylation of Ser62 by PP2A, generates an unstable form of Myc having a single phosphorylation within MB1 on Thr58. This forms a phosphodegron motif, recognized by the FbxW7 subunit of the E3 ubiquitin ligase SCF^{Fbxw7} which, together with cdc34, modifies Myc with Lys48-linked polyubiquitin chains that target it for destruction by the proteasome. This pathway has been the subject of efforts to target Myc therapeutically, using inhibitors of PI3K/Akt/mTOR that are upstream of Gsk3β [6].

Another target in this pathway is the Ser/Thr protein kinase Aurora-A, inhibitors of which are currently under clinical investigation in neuroblastoma [40]. Aurora-A blocks the proteolytic degradation of N-Myc in neuroblastoma cells through binding to the N-Myc/SCF^{Fbxw7} complex, and by reducing the proportion of Lys48 linkages in the poly-ubiquitin chains [41]. Intriguingly, kinase-dead mutants of Aurora-A are also able to stabilize N-Myc, suggesting that Aurora-A must act through a kinase-independent mechanism. Certain Aurora-A inhibitors such as MLN8237/alisertib and CD532 can destabilize N-Myc or c-Myc by disrupting the complex, whilst other Aurora-A inhibitors have little effect [42, 43, 44].

Crystal structure of the Aurora-A/N-Myc complex

Aurora-A is best known for its role in the regulation of mitosis, specifically through the coordination of protein-protein interactions that govern the timing and robustness of the assembling mitotic spindle [45-48]. The catalytic activity of Aurora-A is stimulated upon interaction with the microtubule-associated protein TPX2, which promotes kinase autophosphorylation [49, 50]. Crystal structures of Aurora-A show how this activation process converts the kinase from an inactive to an active conformation [51-53]. Indeed, unlike many kinases that are regulated by activation loop phosphorylation, formation of a fully-active Aurora-A requires both phosphorylation and binding of TPX2 [54]. This is because the activation loop of Aurora-A is particularly dynamic, and requires both these events to lock it into a conformation that is compatible with the binding of substrates [49, 55, 56].

We recently began to investigate the interaction of Aurora-A with N-Myc. We first mapped the regions of the two proteins involved in the interaction, finding that the catalytic domain of Aurora-A interacts directly with N-Myc through binding sites that flank either side of MBI, a region spanning residues 28-89 (Figure 1). We co-crystallized this fragment of N-Myc with the catalytic domain of Aurora-A phosphorylated on Thr288 and determined the structure of the complex [13]. There was clear, unambiguous electron density from residues 61-89, but unfortunately residues 28-60 were unresolved, perhaps due to competition with crystal packing interactions. N-Myc residues 61-89 bind to the interface between the N-lobe and C-lobe of the kinase, interacting with the αC helix, activation loop and αG helix (Figure 2a). This binding site is different from the location of TPX2 binding or the expected site of protein substrates, but there are overlaps (Figure 2b,c). First of all, the pocket on the surface of Aurora-A into which the side chain of N-Myc Leu61 binds is also recognized by the side chain

of TPX2 Trp30. Consistent with this structural overlap, N-Myc competes with TPX2 for binding to Aurora-A. Second, the side chain of N-Myc Trp77 packs into the P+1 pocket of Aurora-A, so called because it accommodates the side chain of the residue one position after the phosphorylated Ser/Thr of kinase substrates. Based on this observation, we thought that N-Myc might block kinase activity or alter the substrate specificity of Aurora-A. Indeed, we found that N-Myc competes with substrate, but does not appear to change the substrate preference of the kinase (S.Burgess, unpublished data).

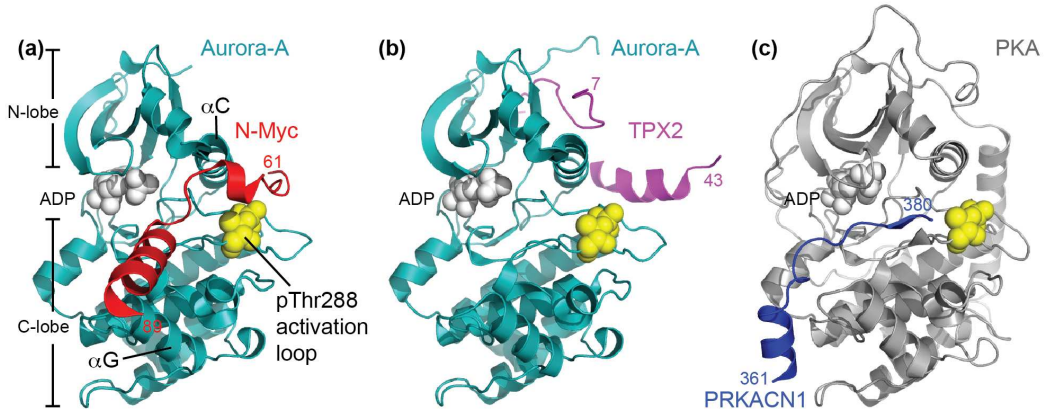

Figure 2. Proteins binding to the N-lobe/C-lobe kinase interface in three different binding modes. (a) Aurora-A (cyan) in complex with N-Myc residues 61-89 (red; PDB entry 5G1X) [13]. (b) Aurora-A (cyan) in complex with TPX2 residues 7-43 (magenta; PDB entry 1OL5) [49]. (c) PKA (grey) in complex with PRKACN1, an inhibitory substrate analogue (blue; PDB entry 1JBP) [57]. ADP is shown as white spheres. Phosphorylated Thr288 in Aurora-A and phosphorylated Thr197 in PKA are shown as yellow spheres.

Somewhat paradoxically, N-Myc dramatically enhances the activity of initially unphosphorylated Aurora-A by stimulating kinase autophosphorylation. Unfortunately, our current structure of the complex does not address this mechanism because residues 61-89 of N-Myc are insufficient to activate the kinase. However, the crystal structure of the Aurora-A/N-Myc complex provides some insight because it shows the kinase in a fully active conformation, essentially identical to that observed for the TPX2/Aurora-A complex (Figure 2). This conformation of Aurora-A is necessary for the formation of the P+1 pocket, to which both the P+1 residue of substrate polypeptides and Trp77 of N-Myc bind. We therefore proposed a molecular switch mechanism in which N-Myc induces Aurora-A phosphorylation to form a stable scaffold on which a region of the N-Myc TAD folds. This stabilizes the conformation of N-Myc and alters the interactions with other proteins that regulate N-Myc stability, and may potentially contribute to the regulation of N-Myc function.

The crystal structure of the Aurora-A/N-Myc complex clarifies the mechanism by which Aurora-A inhibitors such as alisertib and CD532 disrupt the interaction with Myc [13]. These inhibitors promote structural changes in the kinase, twisting apart the two lobes of the kinase and distorting the conformation of the activation loop. Thus, in the presence of these inhibitors, the binding site for N-Myc 61-89 is disrupted. The same 61-89 region of N-Myc that interacts with Aurora-A in the crystal structure was also observed to form a low-affinity interaction with SCF^{Fbxw7}. We proposed that if this interaction were required for specifying K48-linked ubiquitination of N-Myc, perhaps at a site where such modification is crucial for efficient degradation, then competition with Aurora-A for binding to this site may explain the phenomena of Aurora-A-dependent stabilization of N-Myc and destabilization by the compounds that induce Aurora-A conformations with reduced affinity for N-Myc.

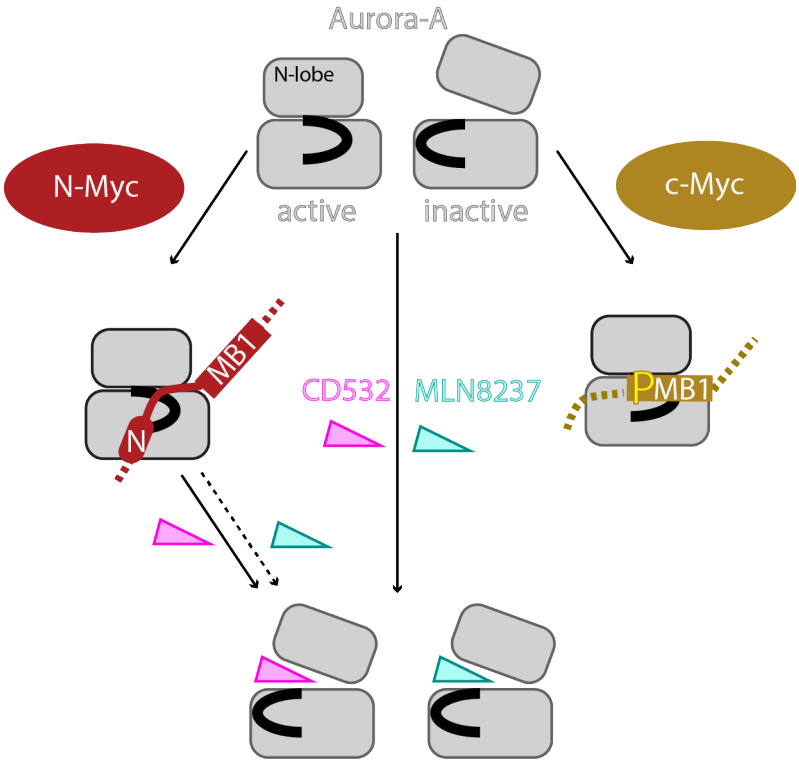

Figure 3. Schematic illustration showing the antagonistic binding of Myc and inhibitors to Aurora-A. Aurora-A can adopt active and inactive conformations, characterized by the position of the N-lobe and activation loop. N-Myc and c-Myc bind to Aurora-A in its active conformation, through overlapping but non-identical regions of the Myc proteins. CD532 and MLN8237 bind to an inactive conformation of Aurora-A, which then blocks the formation of N-Myc or c-Myc to the kinase. CD532 displaces N-Myc from Aurora-A, and so does MLN8237, albeit less efficiently (dashed arrow). In contrast, neither CD532 nor MLN8237 can displace c-Myc from Aurora-A.

Interactions of Aurora-A with c-Myc and L-Myc

Aurora-A also stabilizes c-Myc in hepatocellular carcinoma (HCC) although it is unclear whether the mechanism involves disruption of the c-Myc/SCF^{FbxW7} interaction or alteration of c-Myc ubiquitination [44]. We have confirmed direct interactions with the TADs of c-Myc and L-Myc, but it is not clear how these proteins interact with Aurora-A because the region of N-Myc spanning residues 61-89 is not conserved in c-Myc or L-Myc. Indeed, an *in silico* analysis by Zender and colleagues suggests that c-Myc binds to Aurora-A at a site that overlaps the location we discovered experimentally for N-Myc but predicts contributions from additional Myc residues to the interaction [44]. However, these analyses await experimental verification. While we observed a direct interaction between purified, recombinant, unphosphorylated c-Myc and Aurora-A, and observed no effect of Thr58 and Ser62 phosphorylation upon binding of N-Myc peptides to Aurora-A *in vitro*, Zender and colleagues suggested that c-Myc must be phosphorylated on Thr58 and Ser62 to efficiently bind Aurora-A [44]. Since this was supported by an experiment in which a doubly phosphorylated Myc 46-89 peptide was incubated with cell lysates, the observed binding may have required other cellular factors. For example, there may be another protein that recognises Thr58/Ser62 phosphorylated Myc, and stabilizes its interaction with Aurora-A in a cellular context, but is dispensable for interaction *in vitro* or a protein that binds to the unphosphorylated MBI, blocking the interaction with Aurora-A in cells, and is displaced by MBI phosphorylation, revealing the binding site.

Interestingly, CD532 has an effect on N-Myc levels in neuroblastoma cells within hours, while at least a day is required in the case of c-Myc in HCC and the effects of MLN8237 are slow in both types of cancer cell [43,44]. These differences suggest that c-Myc is more effective than N-Myc in locking Aurora-A in a conformation that cannot be reversed by inhibitors (Figure 3). However, these differences may also reflect the distinct biological contexts of the two tumour types in which the complexes were studied. Further studies are required to elucidate the structural basis of c-Myc/Aurora-A interactions, the role of Myc phosphorylation in complex formation, and the mechanisms that underpin the differential effects of inhibitors in cells derived from neuroblastoma, HCC and other cancer types. The differential activity between CD532 and MLN8237 might be due to differences in their binding to Aurora-A (CD532 induces a more pronounced twist between the N- and C-lobes) or due to differences in off-target inhibition of other kinases. These issues require further study but both MLN8237 and CD532 are, nevertheless, valuable chemical tools for the study of the Aurora-A/Myc interaction.

Conclusion

Myc proteins consist of a series of conserved sequence motifs in the context of an intrinsically disordered protein that adopt ordered conformations only in presence of binding partners. These binding partners have roles associated with transcriptional regulation, chromatin-modification, stability and in many cases are likely to be required for the oncogenic functions of Myc. Inhibiting these interactions with ligands that stabilize Myc in its disordered form are becoming possible and now structures of small, conserved Myc fragments in complex with their binding partners are being resolved using NMR spectroscopy or X-ray crystallography and are providing new insights into Myc biology. The Myc proteins are of huge importance as cancer drug targets, yet their lack of ordered domains has deprived us of pockets that could be targeted in drug discovery initiatives. Structures such as these, which reveal the ordered conformations that sub-regions of Myc can adopt when in complex with other proteins, may allow us to bring structure-based drug design to bear on Myc after all.

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