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Viral mutations enhance the Max binding properties of the vMyc b-HLH-LZ domain

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ABSTRACT

Interaction with Max via the helix–loop–helix/leucine zipper (HLH-LZ) domain is essential for Myc to function as a transcription factor. Myc is commonly upregulated in tumours, however, its activity can also be potentiated by virally derived mutations. vMyc, derived from the virus, MC29 gag-Myc, differs from its cellular counterpart by five amino acids. The N-terminal mutation stabilizes the protein, however, the significance of the other mutations is not known. We now show that vMyc can sustain longer deletions in the LZ domain than cMyc before complete loss in transforming activity, implicating the viral mutations in contributing to Myc:Max complex formation. We confirmed this both *in vitro* and *in vivo*, with loss of Max binding correlating with a loss in the biological activity of Myc. A specific viral mutation, isoleucine$_{383}$>leucine (I$_{383}$>L) in helix 2 of the HLH domain, extends the LZ domain from four to five heptad repeats. Significantly, introduction of I$_{383}$>L into a Myc mutant that is defective for Max binding substantially restored its ability to complex with Max *in vitro* and *in vivo*. We therefore propose that this virally derived mutation is functional by significantly contributing to establishing a more hydrophobic interface between the LZs of Myc and Max.

INTRODUCTION

Retrovirally transduced oncogenes have acquired mutations that considerably potentiate their transforming activity by subverting their normal regulation in a cell. These mutations may result in altered regulation of the oncoprotein by key signalling pathways (e.g. phosphorylation), a reduction or interference in key protein:protein interactions, altered protein turnover or a combination of all these. Indeed, comparative studies between these virally derived oncogenes and their cellular counterparts have contributed greatly toward our current understanding of their molecular mechanism of action. vSrc, the transforming component of Rous sarcoma virus, and vErbB, isolated from the avian erythroblastosis virus, AEV, contain several that contribute to their constitutive tyrosine kinase activity (1,2).

Nuclear oncogenes have also been retrovirally transduced and sustained mutations that potentiate their transforming activity (3–5). Retrovirally transduced cJun and cFos, the two components of the AP-1 transcription factor complex, have sustained mutations which abrogate key phosphorylation events and contribute to an increased half life respectively (3,4).

cMyc, the transforming component of the avian leukosis virus, MC29, belongs to the basic/helix–loop–helix/leucine zipper (b-HLH-LZ) class of transcription factors (6). Somatic and virally derived point mutations in Myc potentiate its function as an oncoprotein, the majority of which are clustered within the transactivation domain at the N-terminus of the protein (7–10). Of the five mutations in MC29 vMyc (11), a functional consequence has only been attributed to threonine 61. This mutation, threonine$_{61}$>methionine (T$_{61}$>M) which is a known phosphorylation site (12), results in significant stabilization of the Myc protein (7,13,14).

Myc functions in association with a small unrelated protein, Max, dimerizing through the C-terminal HLH-LZ domain. Dimerization with Max is not, however, sufficient for Myc to function, since the complex must also be able to bind to a specific target DNA sequence and activate transcription through its N-terminus (6). The LZ domains of Myc and Max form a parallel two-stranded α-helical coil and dictate the specificity of heterodimerization (15). A detailed sequence comparison between the LZs of different transcription factors shows that in contrast to Fos and Jun which each contain five leucine residues in a heptad repeat (denoted L1–L5 in Figure 1), cMyc contains only four (denoted L2–L5 in Figure 1). Strikingly, in MC29 vMyc, mutation of isoleucine

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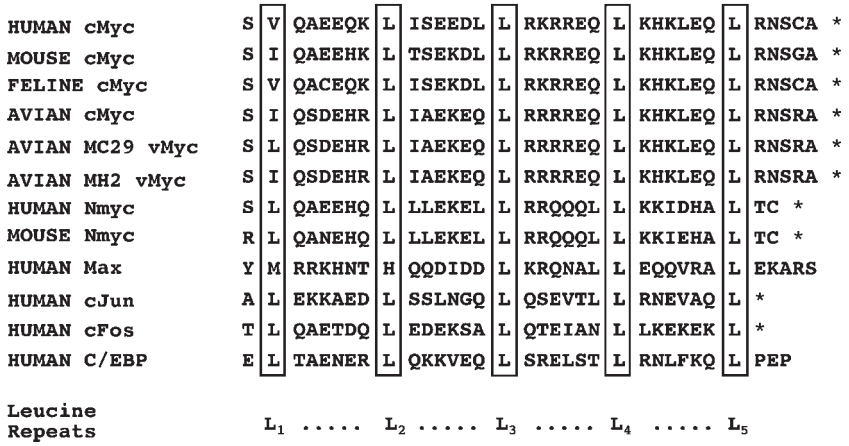

Figure 1. Sequence alignment of the LZ domains of different transcription factors. The amino acids that correspond to position 7 in a heptad repeat are boxed. The leucine repeats are indicated below, with leucine 1 (L_1) and leucine 5 (L_5) being the most N-terminal and C-terminal of the heptad repeats, respectively. Sequences were taken from the following accession numbers: human cMyc (NM_002467), murine cMyc (NM_010849), feline cMyc (M22727), avian cMyc (J00889), avian MC29 vMyc (VO1173), avian MH2 vMyc (K02082), human Nmyc (NM_005378), murine Nmyc (NM_008709), human Max (NM_002382), human cJun (NM_002228), human cFos (BC004490) and human C/EBP (NM_005194).

to a leucine at position 383 (Figure 1) extends the cMyc LZ to five leucine residues in a heptad repeat. This led us to speculate that by increasing the length of the LZ, this virally derived mutation may contribute positively to the interaction with Max. The data we present in this manuscript is consistent with this and for the first time, we propose a functional consequence of this virally derived mutation in the C-terminus of vMyc.

MATERIALS AND METHODS

Cell culture and transfections

Cell culture and transfection of appropriate SFCV-Myc constructs (10 μg) together with RCAN (A) helper (4 μg) into secondary chick embryo fibroblasts (CEFs) was performed essentially as described (16). Following G418 selection, cultures were expanded and used to analyse alterations in cell behaviour which was mediated by overexpression of the Myc oncoprotein (16). Briefly, anchorage independent growth was determined by plating 2×10^5 cells into 0.35% agar and incubated at 41°C for 2 weeks prior to photography. Growth rate was measured by plating 2×10^5 cells in a 35 mm diameter dish, and cumulative cell counts performed each day.

Construction of retroviral vectors expressing mutant Myc alleles

The construction of SFCV-cMyc and SFCV-vMyc has been described (17). All the LZ mutants of vMyc were generated by site directed mutagenesis as described (18) using mp8-vMyc as the template. The mutagenic oligonucleotides were as follows: vMycΔ7, 5′-aaccttgagtagctaaggaag-3′; vMycΔ10, 5′-agttgaaacactaacttgagc-3′ and vMycΔ14, 5′-gtgtttcaactattctctcctccgcctcaa-3′. To generate the isoleucine$_{383}$>leucine leucine (I_{383}>L) mutant, mutagenesis was performed using the primer 5′ gttctgtctccaatcggacgag 3′. To generate the I_{383}>LΔ10 mutant, mutagenesis was performed on the I_{383}>L template using the primer 5′-gttgaaacactaacttgagc-3′. The underscored nucleotides encode the mutant amino acid.

The resulting mutants were retrieved from mp8 and cloned as HindIII fragments into SFCV-sa$^-$ (17) and pSPT19 (19). All mutant sequences were confirmed by double stranded sequencing.

Western blot analysis

Cell lysates were prepared by lysing cultures in SDS-sample buffer (20). Following sonication and protein estimation, 50 μg protein was loaded onto 7.5% SDS–PAGE gels. Transfer to nitrocellulose and western blotting was performed as described (18). Proteins were detected using specific rabbit antibodies [anti-cMyc antibody, 237 (19) and anti-Max antibodies (21)] and visualized using either NBT/BCIP (anti-cMyc 237) or enhanced chemiluminescence (anti-Max).

In vitro translation of Myc and Max proteins and immunoprecipitation

Dimerization between Myc and Max was determined using [^{35}S]methionine-labelled *in vitro* translated proteins essentially as described (19). Briefly, following incubation with Myc proteins, Max or Max9 were specifically immunoprecipitated using an anti-cMyc antibody, 237. The immune complexes were recovered on protein A–Sepharose beads, washed thoroughly and resolved on 10% SDS–PAGE gels. The [^{35}S]methionine labelled proteins were detected by fluorography using Amplify (Amersham).

Reporter and activator plasmids

The PHO5 UAS-CYC-LacZ reporter plasmid, pRS314-Max/Max9, PHO4-cMyc (Pho4-cMyc) and Pho4 cMyc LZ mutant hybrids have been described previously (19). The PHO4-vMyc (Pho4-vMyc), Pho4 vMyc LZ mutants and Pho4-I>LΔ10 hybrids were made by PCR of the appropriate templates and were cloned into the BglII site of pMA132 (19). The integrity of all expression constructs was verified by sequencing.

The Pho5 UAS-CYC-Lac Z reporter plasmid encodes the Pho4 DNA-binding sequence upstream of β-galactosidase. This sequence, CACGTG, has also been shown to be a

consensus sequence for the Myc/Max complex (22). The Pho4-Myc plasmids express the Pho4 transactivation domain fused to the b-HLH-LZ domain (amino acids 327–415) of the different Myc isoforms. pRS315-Max/Max9 expresses the two different Max isoforms.

Dimerization between the b-HLH-LZ domains of Myc and Max was detected by transforming the above plasmids into yeast. The Pho4–Myc/Max complexes formed *in vivo* will bind to the Pho5 UAS-CYC consensus sequence upstream of the LacZ gene. Transcription of LacZ will then be initiated by the Pho4 transactivation domain. The level of β-galactosidase activity in cell lysates of the transformants will therefore be a direct measure of the extent of dimerization between the b-HLH-LZ domains of Myc and Max (19).

The construction of pGV256-lex-OP was as described (23). To generate pRS315-lex-Max9, the Max 9 coding sequence was first inserted as a BglII fragment into pV44ER-lex (23). The entire cassette comprising the GAL UAS, CYC promoter, Lex-Max9 and the CYC terminator was then cloned as an Sst1–Kpn1 fragment into pRS315.

These plasmids were used in an alternate dimerization assay that independently measures Myc/Max complex formation. pGV256-lex-OP contains the Lex operator upstream of CYC-LacZ (23). Lex A, the bacterial repressor, binds to the Lex operator. pRS315-lexA-Max 9 encodes a fusion protein between the bacterial Lex A repressor and Max 9, which will bind to the Lex operator. These plasmids were then transformed into yeast along with Pho4-Myc. Any Lex A-Max9:Pho4-Myc complexes that are formed will bind to the Lex operator sequence via LexA-Max9. The β-galactosidase activity that is detected in the transformants will be the direct result of the complex formation between LexA-Max 9 and Pho4-Myc, since the Pho4 transactivation domain will mediate transcriptional activation. This assay therefore provides a dimerization assay which is independent of DNA binding mediated by the Myc:Max complex.

Yeast culture and β-galactosidase assays

Reporter assays were performed in the yeast strain Y700 (α, his3–11, ade2–1, leu2–3, –112, ura3, trp1–1 and can1–100) essentially as described (19). Following selection on yeast glucose minimal agar plates supplemented with the appropriate amino acids, the transformants were grown in liquid culture and β-galactosidase activity measured (22). Activities represent the average of at least two independent duplicate cultures within the same experiment and units of β-galactosidase activity were calculated as described (19).

RESULTS

vMyc can sustain longer truncations in its LZ domain than cMyc before loss of biological activity

Previous work from our laboratory showed that the integrity of the LZ domain was essential for Myc to function (18). Deletion of seven amino acids (removal of L5) in cMyc resulted in a mutant, cMycΔ7, which retained partial transforming activity (18). Deletion of a further three amino acids (removal of L4a and L5), however, resulted in a non-transforming mutant of Myc, cMycΔ10. We reasoned that vMyc might be able to sustain longer truncations in the LZ domain than cMyc before complete loss of biological activity. To address this, we generated a series of point mutants of vMyc which successively truncated leucine residues from the C-terminus. These mutants, designated vMycΔ7, vMycΔ10 and vMycΔ14, were cloned into avian retroviral vector, SFCV-sa$^-$ (24) and transfected into CEF. It should be noted that these constructs only encode the vMyc portion of MC29, and not the p110 gag-Myc encoded by the original MC29 virus (25). Following G418 selection, expression of all Myc-containing constructs was confirmed by western blot analysis (Figure 2). All were expressed in CEF, although the vMycΔ14 and cMycΔ14

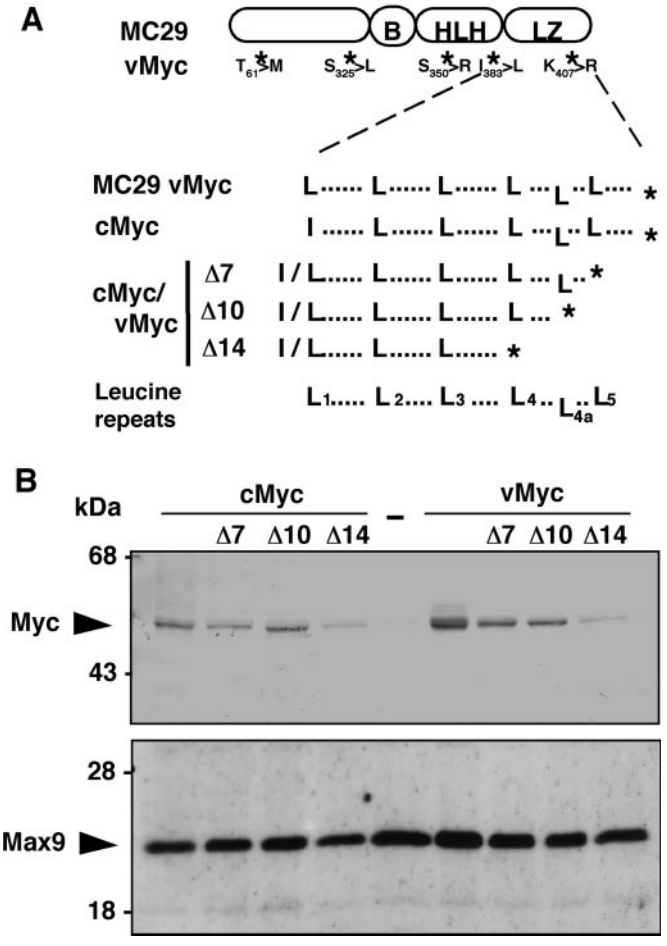

Figure 2. LZ mutants of MC29 vMyc and avian cMyc. (**A**) Schematic representation showing location of MC29 vMyc mutations. Five mutations are contained within MC29 vMyc (11). These are found in the transactivation domain (T_{61}>M), adjacent to the basic region (B) (serine$_{325}$>leucine—S_{325}>L), within helix 1 (serine$_{350}$>arginine—S_{350}>R) and helix 2 (I_{383}>L) of the HLH domain, and within the LZ domain (lysine$_{407}$>arginine—K_{407}>R). LZ deletion mutants of MC29 vMyc and avian cMyc are shown. The leucine repeats (L_1–L_5) within the LZs of MC29 vMyc and avian cMyc are also indicated. Leucine L_{4a} is shown offset to indicate its internal location (position 3) within the most C-terminal heptad repeat. Premature translation termination codons (indicated by an asterisk) were introduced into MC29 v-*myc* and avian c-*myc* (18) by site-directed mutagenesis. The Δ7 and Δ14 mutants truncate specifically at L_5 and L_4 within the heptad, respectively, whilst the Δ10 truncation removes L_5 and L_{4a}. (**B**) Retrovirally-expressed cMyc, vMyc and their respective LZ mutants in CEFs were detected by western blot of total cell lysates using an anti-Myc antibody (upper panel). Equivalence of loading was shown by Max9, the main isoform of Max in CEF (17,26), which was detected using an anti-Max antibody (21) (lower panel). Vector control is shown by a dash.

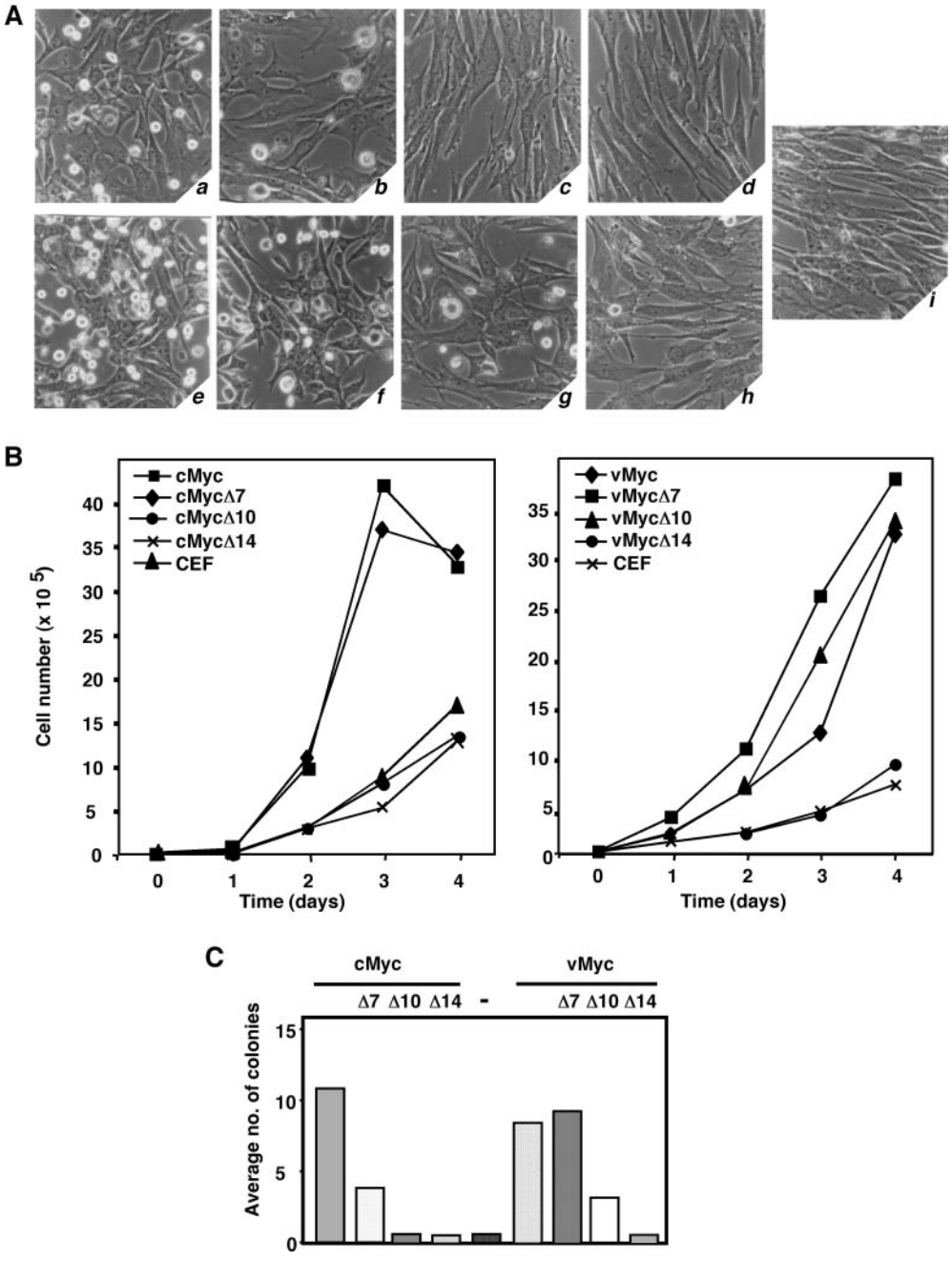

Figure 3. vMyc can sustain longer deletions in the LZ than cMyc before loss of biological activity (**A**) Cell morphology of CEF expressing cMyc, vMyc and their respective LZ mutants are shown. (a) cMyc, (b) cMycΔ7, (c) cMycΔ10, (d) cMycΔ14, (e) vMyc, (f) vMycΔ7, (g) vMycΔ10, (h) vMycΔ14 and (i) vector control. (**B**) Growth rate of CEF expressing cMyc, vMyc and their respective LZ mutants. Growth rate was measured by cumulative cell counts over 4 days. These data are representative of at least two different experiments. (**C**) The ability of retrovirally-expressed cMyc, vMyc and their respective LZ mutants to induce anchorage-independent growth was determined by plating infected CEF into soft agar. Colony counts were taken after 14 days. Vector control is shown by a dash. These data are representative of at least two different experiments.

mutants did appear to be expressed at lower levels than the wild-type. These low levels were unexpected. These mutants should bind Miz-1, but not Max (Figures 4 and 5). Since the former stabilizes Myc, and the latter has no affect on its half life (13), we would expect at least equal levels of the vMycΔ14 and cMycΔ14 mutants. Chicken Miz-1 has not, however, been characterized, and its levels in CEF are not known. The significance of the reduced expression of these two mutants is therefore not known. The level of Max9, the major Max isoform in CEF (17,26), does not change in the Myc-infected cells (Figure 2B).

CEF which overexpress Myc undergo extensive changes in cell morphology, grow more rapidly and acquire the ability to grow in an anchorage independent manner (16,18). Therefore, having confirmed the appropriate retroviral expression, we determined changes in cell morphology (Figure 3A), calculated the growth rate (Figure 3B) and determined the ability to grow in agar of CEF overexpressing each Myc mutant (16,18).

Control chick cells are spindle shaped, non-refractile and contact inhibited, lining up in a parallel manner on the dish (Figure 3A, i). In contrast, cMyc-infected CEF are rounder, more refractile and do not undergo contact inhibition (Figure 3A, a). cMycΔ7 (Figure 3A, b) had an intermediate phenotype, being partially morphologically-transformed. Deletion of 10 amino acids or more from the cMyc LZ, however, resulted in mutants that were non-transforming (Figure 3A, c and d). vMyc (Figure 3A, e) was more highly transformed than cMyc (Figure 3A, a), highlighting a major role for the viral mutations in potentiating the biological activity of Myc (9). vMyc (Figure 3A, e) and vMycΔ7 (Figure 3A, f) were morphologically indistinguishable from each other and were highly transformed. vMycΔ10 (Figure 3A, g) was partially transformed, however, vMycΔ14 (Figure 3A, h) was non-transformed. Thus, deletion of 14 amino acids from the vMyc LZ was required before loss in biological activity, whilst deletion of only 10 amino acids was required before cMyc became functionally inert. These data clearly show that vMyc can sustain longer truncations in its LZ than cMyc before loss of morphological transformation.

Another feature of Myc-transformed cells is an accelerated cell growth (18). The growth rate of Myc-infected CEF, determined by cumulative cell counts >4 days, is shown graphically (Figure 3B). As previously reported, cMyc and cMycΔ7 have accelerated growth rates, but further truncation of the LZ domain resulted in growth rates comparable with uninfected CEF (18). In contrast, vMyc, vMycΔ7 and vMycΔ10 grew more rapidly than control cells, whilst vMycΔ14 was indistinguishable from the control.

Loss of anchorage dependence by transformed cells was measured by the ability to grow in soft agar. Myc-infected CEF were plated in agar and after 2 weeks, plates were photographed. A minimum of eight frames were taken for each and the average number of colonies calculated (Figure 3C). It can clearly be seen that in contrast to vMycΔ7, cMycΔ7 only partially retains the ability to grow in agar. In contrast to cMycΔ10, vMycΔ10 was however still partially able to grow in agar.

Collectively, these data show that the integrity of the LZ domains of vMyc and cMyc are required for biological activity. More importantly, these results highlight a major functional difference in the behaviour of the vMyc and cMyc LZ domains, since vMyc can sustain longer truncations than cMyc before complete loss in its biological activity (Table 1). Indeed, deletion of 10 amino acids from the C-terminus of vMyc results in a mutant that still retains biological activity, whilst deletion of 10 amino acids from cMyc results in a mutant that is functionally inactive. This difference in the behaviour of the LZ domains must be a direct consequence of the virally derived mutations.

Table 1. LZ mutants of avian cMyc and vMyc are functionally different

	Morphology	Anchorage independent growth	Growth rate	Dimerization with Max
cMyc	+	+	+	+
cMyc Δ7	±	±	+	±
cMyc Δ10	−	−	−	−
cMyc Δ14	−	−	−	−
vMyc	++	++	+	+
vMyc Δ7	++	++	+	+
vMyc Δ10	±	±	+	±
vMyc Δ14	−	−	−	−

vMyc can sustain longer truncations in its LZ before loss in biological activity and binding to Max. Summary of the biological activity of LZ mutants of avian cMyc and vMyc expressed in CEFs. vMyc can sustain longer deletions of the LZ before complete loss in biological activity. Like cMycΔ7, vMycΔ10 retains partial biological activity, whilst cMycΔ10 is biologically inactive.

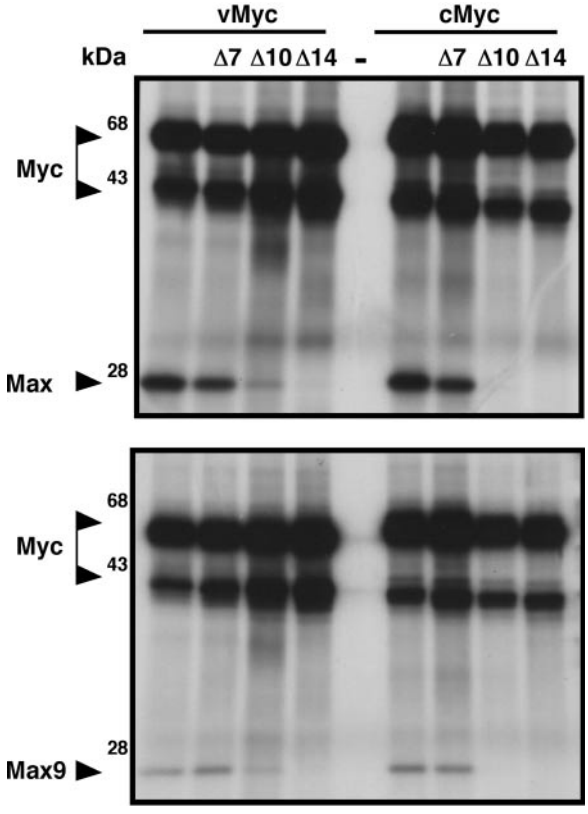

Figure 4. Complex formation between Max/Max9 and the LZ mutants of v- and c-Myc *in vitro*. cMyc, vMyc, LZ mutants and Max/Max9 were produced in rabbit reticulocyte lysate and Myc/Max complex formation determined by immunoprecipitation of Max using an anti-*myc* specific antibody. [^{35}S]-labelled proteins were resolved by SDS–PAGE and visualized by fluorography. Central lane was Max/Max9 alone.

Biologically active LZ mutants of vMyc retain the ability to dimerize with Max *in vitro* and *in vivo*

Since Myc requires Max to function (27), we set out to establish whether the vMyc LZ mutants retained the ability to dimerize with Max. [^{35}S]-labelled Myc and Max were produced in an *in vitro* translation system, and Myc–Max complexes allowed to form at 37°C. Complexes were recovered by immunoprecipitation using an anti-Myc antibody that does not recognize Max (Figure 4). The central lane in both panels, indicated by a dash, contained Max or Max9 alone demonstrating that the Myc antibody does not immunoprecipitate Max. From these data, it can be seen that in contrast to vMyc, vMycΔ7 and vMycΔ10 which can complex with Max (upper panel) and Max9 (lower panel), only cMyc and cMycΔ7 complex with Max. Therefore, these data confirm that

vMyc can sustain greater truncations to the LZ domain than cMyc before losing the ability to complex with Max. Importantly, all the transforming mutants of both vMyc and cMyc (Figure 3) bound to Max and Max9.

To confirm these data, we tested the ability of the LZ mutants of Myc to complex with Max *in vivo* using a yeast assay designed to measure dimerization and DNA binding of Myc:Max complexes. This assay has been used previously as a reliable and sensitive assay for Myc:Max function *in vivo* (19,28). In this assay, the bHLH-LZ domain of avian Myc or vMyc (amino acids 330–417) or their respective LZ mutants, were fused to the transactivation domain of the yeast transcription factor Pho4. When Pho4-Myc was transfected into yeast together with a PHO5 UAS-CYC-lacZ reporter plasmid, transcription through the PHO5-UAS occurred only in the presence of Max or Max9 (19). As expected, only Pho4-cMyc and Pho4-cMycΔ7 were able to activate transcription, whilst Pho4-cMycΔ10 and Pho4-cMycΔ14 did not. In contrast to cMyc, co-expression of Max or Max9 with Pho4-vMyc, Pho4-vMycΔ7 and Pho4-vMycΔ10 activated transcription through the PHO5-UAS, albeit at a reduced level for Pho4-vMycΔ10 (Figure 5A). These *in vivo* data confirm the *in vitro* results (Figure 4) clearly showing that vMyc can sustain a larger truncation to the LZ domain than cMyc before losing the ability to bind to Max.

As an independent measure of dimerization, we used an assay which asks whether Myc function was mediated by direct complex formation with Max, independently of its DNA binding activity. To achieve this, we fused the bacterial protein, LexA, to Max9, generating pRS315-lexA-Max9. When transfected into yeast, LexA binds to a specific DNA sequence, LexOP, which lies upstream of β-galactosidase, however, no β-galactosidase activity will be detected in the absence of Pho4-Myc. As expected, no activity was detected when pRS315-lexA-Max9 was transfected into yeast along with pGV256-lex-OP (23). When they were co-transfected with Pho4-Myc plasmids, however, β-galactosidase activity was recorded. As can be seen from Figure 5B, significant β-galactosidase activity was recorded with all the transforming mutants of cMyc and vMyc.

Collectively, these data (Figures 4 and 5) clearly show that the LZ domains of vMyc and cMyc differ significantly in their ability to bind to Max both *in vitro* and *in vivo*.

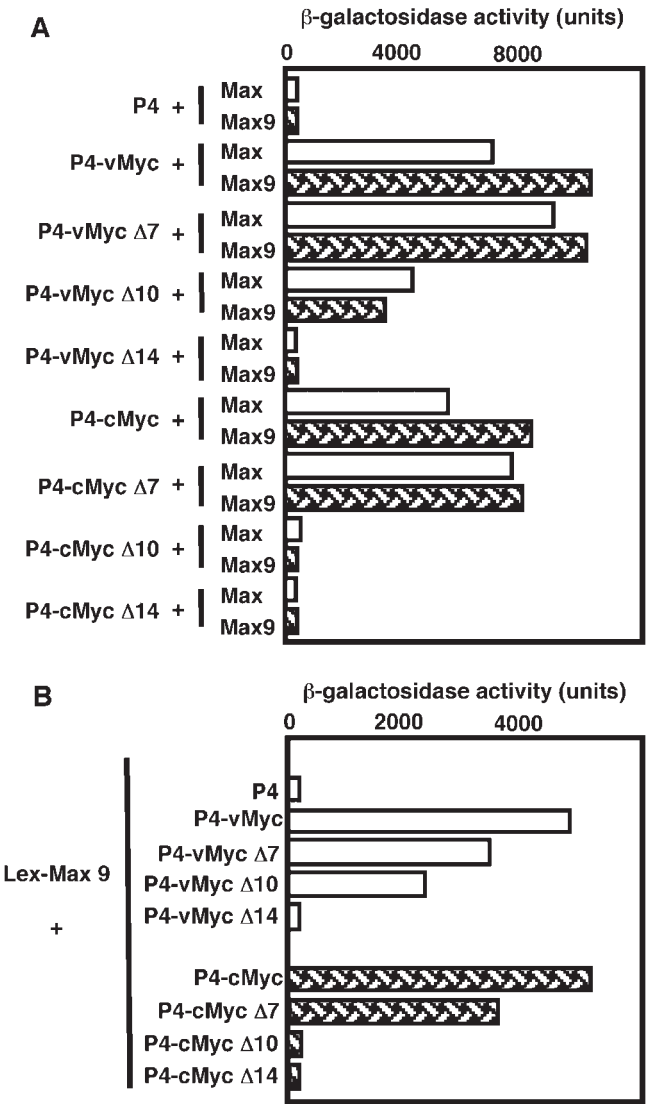

Figure 5. Complex formation between Max/Max9 and the LZ mutants of v- and c-Myc in yeast. Two different experimental systems based on sensitive yeast two hybrid assays were used to measure complex formation *in vivo*. (**A**) Max/Max9-dependent dimerization and DNA binding were determined *in vivo*. β-Galactosidase activity was quantitatively measured as a result of transcriptional activation resulting from the co-transfection of Pho4-cMyc, Pho4-vMyc or their respective LZ mutants into yeast, together with the PHO5 UAS-CYC-LacZ reporter. This assay has been shown previously to a reliable assay for Myc/Max complex formation (28). (**B**) Dimerization which is independent of Myc/Max DNA binding was measured by co-transfecting Pho4-cMyc, Pho4-vMyc or their respective LZ mutants into yeast, along with the reporter, pGV256-lex-OP (23) and pRS315-lexA-Max9. β-Galactosidase activity was then determined.

Mutation of I_{383}>L in cMycΔ10 background partially restores binding to Max *in vitro* and *in vivo*

cMycΔ10 does not bind Max/Max9 (19) (Figures 4 and 5). Theoretically, the I_{383}>L mutation of vMyc could extend the LZ domain from four to five heptad repeats and stabilize the Myc/Max interaction (Figure 1). To directly test this, we introduced this mutation into a cMycΔ10 background. This mutant, cMycI>LΔ10 (Figure 6A) was tested for the ability to complex with Max both *in vitro* (Figure 6B) and *in vivo* (Figure 6C). In both these assays, replacement of isoleucine 383 with a leucine significantly restored binding to both Max and Max9. This mutant did not, however, significantly induce a biological phenotype when overexpressed in CEF, suggesting that the levels of cMycI>LΔ10:Max complex may be below the threshold required to mediate a biological response. These data do, however, show that this single mutation is sufficient to contribute positively to the interaction of Myc and Max.

DISCUSSION

Somatic or virally derived mutations that potentiate the function of a protein can be viewed as naturally occurring protein engineering. Currently, many examples of these exist, which can result in proteins with altered properties, such as different half lives, differential responses to signalling pathways

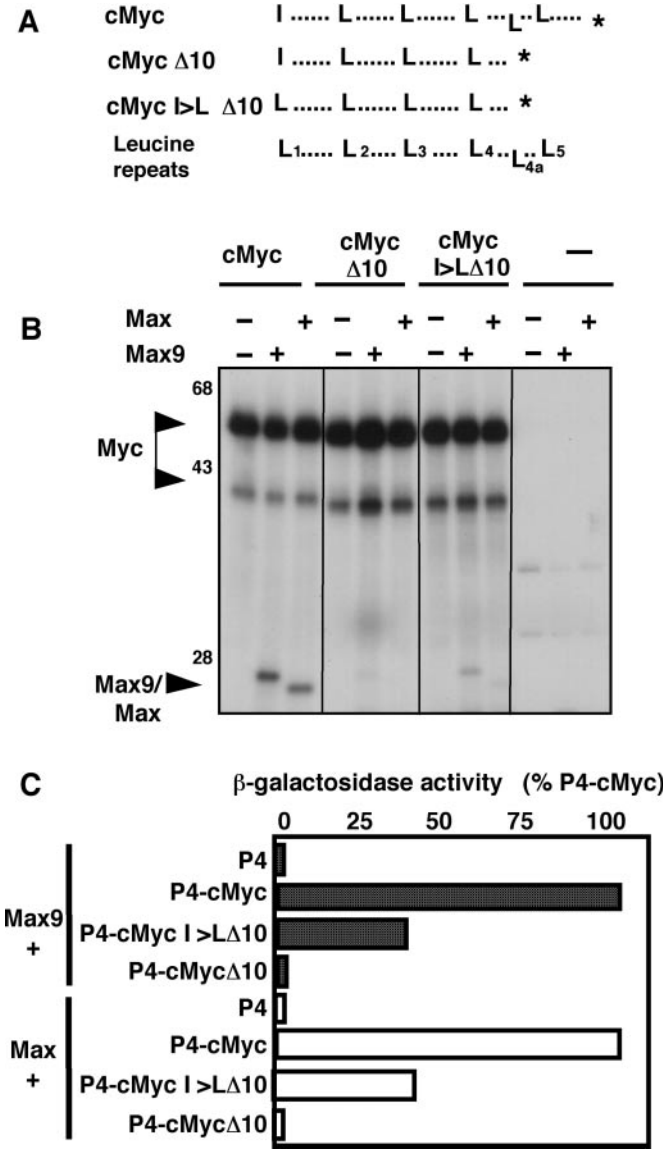

Figure 6. I_{383}>L mutation in cMycΔ10 partially restores interaction with Max/Max9 *in vitro* and *in vivo*. (**A**) Schematic representation of cMyc, cMycΔ10 and cMycI>LΔ10 mutants. I_{383}>L mutation was introduced into cMycΔ10, which does not bind to Max (18). (**B**) Complex formation between Max/Max9 and cMyc, cMycΔ10, and cMyc I>LΔ10 co-translated *in vitro* was determined by immunoprecipitation of Max/Max9 using an anti-Myc antibody, and [^{35}S]-labelled proteins resolved by SDS–PAGE and visualized by fluorography. (**C**) Complex formation between Max/Max9 and Pho4-cMyc, Pho4cMycΔ10 and Pho4-cMycI>LΔ10 was measured *in vivo* by co-transfecting the appropriate plasmids into yeast. β-Galactosidase activity was then determined.

through loss in phosphorylation sites or altered protein:protein interactions. In the data presented here, viral mutations clearly enhance the Max binding properties of the b-HLH-LZ domain, since the vMyc LZ can tolerate greater loss of its dimerization interface than cMyc before loss of Max binding.

We propose that the I_{383}>L mutation in MC29 vMyc represents an example of naturally occurring protein engineering. Given that HLH-LZ heterodimers are stabilized by hydrophobic and polar interactions involving the α-helices, H1, H2 and LZ (29), the function of the I_{383}>L mutation in the C-terminus of vMyc would be to contribute to establishing a more hydrophobic interface which stabilizes the Myc–Max complex.

Our data are wholly consistent with this proposal, since the introduction of a single amino acid change, I_{383}>L, into cMycΔ10, a LZ mutant which is defective for Max binding, could significantly restore its ability to complex with Max both *in vitro* and *in vivo*. This amino acid substitution did not, however, restore biological activity, although small colonies were detected in agar (data not shown). Therefore, although this mutation contributed positively to the Myc:Max interaction, it is most likely that the levels of cMyc I> LΔ10:Max complexes formed in CEF were below the threshold required to elicit any biological response (17).

The I_{383}>L mutation is located in the C-terminal HLH domain, a region which is known to bind other proteins (30). This mutation may therefore regulate the interaction of Myc with these factors. This is thought highly unlikely given the extremely tight correlation between the ability of the different LZ mutants to bind to Max and transform cells, suggesting that it is the Myc:Max complex which is regulated by these mutations (19,27).

The behavioural differences between the respective LZ mutants of vMyc and cMyc clearly show that the virally derived mutations in vMyc impinge on the structure and function of its b-HLH-LZ domain. Whilst the integrity of the LZ domain of vMyc was still required for its biological activity, the v- and cMyc LZ mutants differ significantly with respect to both their biological activity and their ability to bind to Max (Figures 3–5). Given that the vMyc LZ can tolerate larger truncations from its LZ zipper domain than cMyc before loss in its biological activity and binding to Max, this must reflect a stronger interaction between the LZ domains of vMyc and Max than the LZ domains of cMyc and Max.

These findings could have major implications in the rational design of peptide inhibitors for cancer treatment, since a knowledge of the avidity of protein:protein interactions mediated through HLH-LZ dimerization could dictate the design of peptide inhibitors for use in a therapeutic context. Indeed, one such strategy was recently described which was based on the use of helix 1 (H1) peptides to inhibit the protein:protein interactions between Myc and Max resulting in a block to Myc-mediated cell proliferation (31). Although this study focussed on H1 rather than LZ peptides to block Myc function, it highlights the general applicability of targeting any essential dimerization interface with a view to inhibiting function. Our data could therefore have important implications if the target is a cancer cell which contains a mutated Myc that has a stronger dimerization interface than the wild-type Myc, since therapeutic targeting of this interface with a relatively weak peptide inhibitor would obviously be counterproductive.

Conversely, the stability of LZ interactions could be exploited to therapeutically intervene with Myc:Max complexes in cancer cells. An example of this was described by Jean-Francois *et al*. (29), who introduced two point mutations in Max, His_{82}>Leu and Asn_{78}>Val, with a view to increasing the hydrophobic interface between the two contributing Max monomers. This situation is similar to that described here, since the His_{82}>Leu mutation increased the number of heptad repeats in the LZ from three to four. Their rationale was that the stabilized mutant Max homodimers would compete with Myc:Max heterodimers, block their binding to target E-boxes

and as such, would act effectively as an anti-Myc drug (29). Indeed, this mutant dimer was subsequently shown to have improved thermodynamic stability and form more stable E-box complexes. Therefore, rather than titrating out Max to abolish Myc function (29), Myc peptides that provide a stronger dimerization interface could also theoretically be used to dominantly interfere with the formation of Myc:Max complexes. Obviously, this would only be applicable in a cancer cell that contained wild-type Myc.

In summary, we have shown for the first time a major difference between the C-termini of vMyc and cMyc, since vMyc can sustain greater truncations of the C-terminal LZ before loss in biological activity. Furthermore, we show that a single point mutation in helix 2 from vMyc can in isolation positively contribute to the interaction with Max. We therefore propose that this mutation provides a more hydrophobic surface between the dimerization interfaces of Myc and Max. Together with data showing the functional significance of a mutation in the transactivation domain (7), these data highlight the importance of virally derived mutations as examples of naturally occurring protein engineering.

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