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New insights into the Orange domain of E(spl)-M8, and the roles of the C-terminal domain in autoinhibition and Groucho recruitment

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Abstract

CK2 is a Ser/Thr protein kinase that regulates the activity of the *Drosophila* basic-helix-loop-helix (bHLH) repressor M8 encoded by the *Enhancer of split Complex (E(spl)C)* during neurogenesis. Specifically, phosphorylation appears to elicit a conformational change in an autoinhibited state of M8 to one that is permissive for repression. We describe biochemical and molecular modeling studies that provide new insights into repression by M8. Our studies implicate the phosphorylation domain in autoinhibition, and indicate that binding of the co-repressor Groucho (Gro) is context-dependent. Molecular modeling indicates that the Orange domain, proposed to be a specificity-determinant, may instead play a structural role, and that a conformational rearrangement of this domain may be necessary for repression. This model also provides a structural mechanism for the behavior of mutant alleles of the *m8* gene. The insights gained from these studies should be applicable to the conserved metazoan bHLH repressors of the Hairy and Enhancer of Split (HES) family that are related to *Drosophila* M8.

Keywords

CK2; bHLH; E(spl)C; HES; Orange-domain

Introduction

Recent studies from our laboratory indicate a role for protein kinase CK2 during patterning of two sensory organs in the *Drosophila* model organism. These studies have focused on the number and precise positioning of the mechanosensory bristles [1], and the stereotyped development of the compound eye [2]. The development of both sensory systems requires precise cell-cell communication via the Notch signaling pathway (reviewed in [3, 4]).

During early neurogenesis, groups of cells called proneural clusters (PNCs) are formed in response to the expression of bHLH transcriptional activators [5, 6], such as Achaete-Scute (ASC) or Atonal (Ato). The presence of these activators is necessary to maintain neural potential in cells of the PNC. Later in development, the levels and activity of ASC or Ato are rapidly extinguished in most cells of a PNC, and the single cell that continues to express these activators then goes on to become the sensory organ precursor (SOP). This restriction in the expressivity of ASC or Ato only to the SOP requires juxtacrine cell-cell communication via Notch signaling [7–10]. In this case, high levels of ASC or Ato in the

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presumptive SOP lead to the expression of the ligand Delta at a level sufficient to activate the Notch receptor only in adjacent cells. This directional signal culminates in the transcription of the *E(spl)C*, a locus that encodes seven structurally similar bHLH proteins [11–15]. However, unlike ASC or Ato, the E(spl)-bHLH proteins are transcriptional repressors. The accumulation of E(spl) proteins in the non-SOP cells leads to inhibition of ASC or Ato activities, thereby extinguishing their potential to also become SOPs. This view is consistent with the observations that the loss of E(spl) members leads to the over-specification of SOPs from each PNC, whereas increased E(spl) activity leads to extinction of the SOP, itself (reviewed in [16, 17]). This signaling mechanism, termed lateral inhibition [18, 19], operates in other developmental contexts such as myogenesis and cardiac development, and plays a conserved role in mammalian development [20, 21]. It is therefore important to better define the mechanisms by which E(spl) proteins inhibit ASC or Ato.

All E(spl) proteins contain a number of conserved domains. The N-terminal basic domain mediates binding to DNA-sequences called the N-boxes, and this interaction is thought to play a context-specific role in repression of target genes. It has been proposed that the first HLH domain mediates dimerization amongst the seven E(spl) proteins [22], whereas the Orange (also an HLH) serves as a specificity determinant [23], mediating interaction with Ato and Senseless [24, 25]. The C-terminal WRPW tetrapeptide, also present in Hairy and Deadpan, binds the co-repressor Gro (reviewed in [26, 27]), and this motif has been proposed to be necessary and sufficient for this interaction [28]. While these domains may appear to be modular, studies indicate that inter-domain interactions, which are influenced by phosphorylation, regulate repression in vivo.

Studies during eye development were the first to indicate that repression by M8 is regulated in vivo. Even though the endogenous *m8* gene is expressed during early eye development [29], forced over-expression of an *m8* transgene does not antagonize Ato and block specification of the R8 photoreceptor [2, 29], a founding retinal cell type that is selected from PNCs in a manner akin to the SOP (reviewed in [30–32]). In contrast, a unique dominant mutation of *m8*, called *E(spl)D*, elicits a severe loss of the R8 cells [25]. Since the R8s orchestrate the recruitment of all secondary retinal cell fates, loss of these 'founding' cells leads to a loss of the eye. This dominant mutation eliminates all sequences after the Orange domain, and encodes a truncated protein called M8*. Despite its inability to bind Gro, M8* potently antagonizes Ato via direct interactions. However, no such effect is seen upon removal of just the WRPW motif from full-length M8. To reconcile this paradox, it was suggested that residues between the Orange domain and WRPW likely play an important role in regulating repression [33].

It was subsequently found that protein kinase CK2 modifies M8 at Ser¹⁵⁹ [34]. Forced expression of a variant of M8 that harbored an Asp residue in place of the CK2 phosphoacceptor, M8-S¹⁵⁹D, elicited potent antagonism of Ato, and a dominant loss of the R8 cells [2], effects that mimicked those of *E(spl)D* or M8* (see above). Moreover, the CK2 mimetic variant M8SD also interacted strongly with Ato, with a binding strength equivalent to that of M8* [2]. Consequently, we proposed that non-phosphorylated M8 is autoinhibited. In this regulatory mechanism, the C-terminal domain (CtD) of M8 binds and occludes the HLH/Orange domains, thereby preventing the latter from interaction with Ato (Fig. 1a). Modification by CK2 would displace this inhibitory (*cis*) interaction to allow for binding to Ato. The absence of this inhibitory domain in M8* likely mediates the dominant eye defects of *E(spl)D*. Further support to this model was the finding that the isolated CtD could impair Notch signaling in vivo, and negate the dominant activity of the non-inhibited proteins M8* and M8-S¹⁵⁹D [35]. Together, these studies implicated inter-domain interactions in repression by M8.

To further define the contributions of the CtD in the regulation of protein-protein interactions, we have generated a number of truncations in this region of the M8 protein (Fig. 1b). These variants have been analyzed for interaction with CK2, Gro and Ato. These studies implicate a conserved multi-site phosphorylation domain in autoinhibition, and indicate that Gro-binding by the WRPW motif is context-specific. In addition, using the (unpublished) known structure of the related human protein HESR1 (PDB file 2DB7), we have modeled the Orange domain of M8. These studies indicate that the Orange domain of M8 is more likely an extended dimerization interface. Furthermore, our analysis leads us to argue that residues of Orange previously implicated as determinants of target protein specificity [24] may instead serve a structural role. Further support to the modeling studies is our finding that a unique revertant of *E(spl)D*, which has lost the ability to antagonize Ato and abolish eye development, replaces a highly conserved Gly residue (with Asp) that lies at the interface of the Orange dimer. Importantly, this Asp substitution abrogates dimerization of M8*, indicating that the dominant effects of this protein reflect antagonism of Ato in a dimeric state. We suggest that regulated inter-domain interactions and dimer formation are key to neural repression by E(spl) proteins.

Materials and Methods

Construction of deletion variants of E(spl)-M8

Variants of M8 harboring deletions of regions of the C-terminal domain were generated by inverse-PCR, whereas site-specific variants were generated by conventional PCR-based mutagenesis. All constructs were sequenced to confirm the presence of only the intended mutations. Unless indicated, all constructs were generated with an in-frame EcoRI site at the 5' end and a BamHI site at the 3' end. The construction of the deletion variant M8* has been previously described [25].

Protein-protein interactions

Interactions of M8-variants with CK2 α , CK2 β , and Gro were assessed using a mating two-hybrid system [36]. M8-variants were expressed as fusions with the DNA-binding domain of Gal4 in the vector pGBT9, whereas CK2 α , CK2 β , and Gro were expressed as activation-domain fusions in the vector pACT. Interactions were assessed in yeast strains PJ69-4a and PJ69-4 α , and induction of reporter genes was assessed on selective dropout media.

Formation of M8 dimers and interactions of M8-variants with Atonal were assessed in the LexA-based version of the interaction trap [37]. For dimerization assays, M8 variants were expressed as fusions with the DNA-binding domain of LexA in the vector pEG202, and as fusions with the activation domain of B42 in the vector pJGAB (a derivative of plasmid pJG4-5, [37]). Interactions of M8-variants were tested against AD-Gro or AD-CK2 α (VP16), or involved a B42-Atonal fusion. Interactions were assessed in yeast strain EGY048 containing the *LacZ* reporter plasmid pSH18-34 [37]. For quantitative assessment of interactions, three independent transformants, each in triplicate, were assayed for *LacZ* activity employing ONPG as a substrate as described [34]. *LacZ* activity was determined using the formula $1000 \times OD_{420} / (T \times V \times OD_{600})$, where T is minutes and V is the concentration factor of the assay.

Molecular modeling

The Swiss PDB Viewer (www.expasy.org) and PyMOL (www.pymol.org) software packages were used to model the Orange domain of M8 based on the crystal coordinates of the related human protein HESR1 (PDB file 2DB7). The Gly86 residue of M8 was 'mutated' to Asp to determine potential effects on the structure of the Orange-dimer, and the locations of the EVS and THL motifs were mapped similarly.

Results and Discussion

The C-terminal domain (CtD) and repression by M8

Repression by M8 requires Gro recruitment, and involves interaction of the Orange domain of M8 with Ato. The Orange domain has also been reported to mediate interaction with the zinc-finger protein Senseless (Sens, [38]), a transcriptional target of Ato during the formation of sense organs [24]. In the case of the M8-Ato interaction, *in vivo* and *in vitro* evidence indicate that complex formation requires prior modification of M8 at a highly conserved site for phosphorylation by CK2 (S¹⁵⁹DCD) located in a region called the CtD [2]. Specifically, phosphorylation appears to drive a conformational change in the autoinhibited state of M8 (Fig. 1a), wherein displacement of the CTD exposes the Orange domain, thereby permitting interaction with Ato. In addition, M8 is known to form homo/hetero-dimers [22, 39] through the HLH domain. Loss of this function compromises repression *in vivo* [33], presumably reflecting an inability to bind to Ato or Sens. It is currently unknown if dimer formation occurs prior to, or after, Gro binding. Moreover, it has been suggested that the WRPW motif is necessary and sufficient for Gro recruitment and repression [28]. However, it has remained unknown whether the CtD only serves to auto-inhibit M8, or does it also regulate Gro binding. To better define the potential contributions of the CtD, we subjected this region to deletion analysis, and characterized these variants (see Fig. 1b) for protein-protein interactions.

The CK2 consensus site mediates interaction with CK2 α

We first employed the mating two-hybrid assay [36] to test for protein interactions. We find that diploids co-expressing full-length M8 or the CtD in combination with *Drosophila* CK2 α exhibited robust growth on media lacking adenine (Ade⁻, see Fig. 2). In contrast, no interactions were detected with CK2 β , indicating that this regulatory CK2 subunit was dispensable. The lack of an interaction does not reflect an absence of expression of *Drosophila* CK2 β (in yeast), because it robustly interacts with CK2 α [40, 41]. Co-expression of M8*, a variant lacking the CtD and its resident phosphorylation domain, with CK2 α did not result in any detectable growth on Ade⁻ media, indicating the absence of an interaction (not shown). Thus the basic, HLH or Orange domains do not contribute to the M8-CK2 interaction. We next tested and found that removal of sequences between the S¹⁵⁹DCD and WRPW motifs, i.e., M8-CCK2, did not attenuate the strength of binding to CK2 α . However, further progressive deletions in the Ser-rich phosphorylation domain that removed the CK2 site (S¹⁵⁹DCD), i.e., M8-SPAGYH, or the putative secondary phosphorylation site (S¹⁵¹PASSGY), i.e., M8-NSPAS, abolished interaction (Fig. 2). We conclude that the S¹⁵⁹DCD motif in M8 largely mediates the M8-CK2 α interaction, with minimal contributions from flanking CtD residues.

The interaction of the WRPW motif with Gro appears context-specific

Because the phosphorylation domain is located within 13 residues of the Gro-binding site, we next tested if placement of the WRPW at other positions within the CtD affected interaction with Gro. As previously described by others and us, full-length M8 and the isolated CtD interact robustly with Gro (Fig. 2, and see [28, 29, 35, 42, 43]). However, placement of the WRPW motif immediately C-terminal to the CK2 site, S¹⁵⁹DCD (see Fig. 1b), or to the secondary phosphorylation site SPASSGYH¹⁵⁸ abrogated interaction with Gro (Fig. 2). In contrast, placement of the WRPW motif immediately after Leu¹⁵⁰ (see Fig. 1b), thereby removing the entire phosphorylation domain, i.e., M8-NSPAS, restored interaction with Gro (Fig. 2). It would thus appear that recruitment of Gro by the WRPW motif is impaired when it is juxtaposed to the phosphorylation domain. Sequence alignment of M8 homologues from 13 *Drosophila* species and those members (M8, M5 and M7) that are modified by CK2 indicates that the phosphorylation domain is invariably separated from

WRPW by 12–13 residues (not shown). Given the role of the CtD in M8 autoinhibition, this may reflect the optimal length to support both autoinhibition and Gro recruitment. It thus appears that Gro binding by WRPW is context-specific.

Autoinhibition appears to involve the phosphorylation domain

We next sought to test M8-variants for autoinhibition. For this analysis, we tested for interactions with Ato, using the LexA-based two-hybrid approach. As previously reported, full-length M8 does not interact with Ato, because the low levels of *LacZ* activity (<5 Miller Units) are indistinguishable from the baseline (Fig. 3 and [2]). In contrast, M8*, which lacks the CtD, interacts robustly (Fig. 3), and equivalent levels of *LacZ* were detected with the CK2 phosphomimetic variant M8-S¹⁵⁹D (data not shown). We next compared the relative strength of binding of deletion variants of M8 with Ato using these two values for comparison. We find that variants of M8 containing an intact phosphorylation site (M8-CKK2), or one lacking only the CK2 motif (M8-SPAGYH), did not exhibit any detectable interaction with Ato (Fig. 3). In contrast, removal of the complete phosphorylation domain (M8-NSPAS) resulted in strong interaction with Ato. We conclude that the phosphorylation domain, S¹⁵¹PASSGYHSDCD (see Fig. 1b), is likely to be a major determinant of autoinhibition.

The Orange domain is an extended dimerization domain

Previous studies have led to the view that Orange is a specificity determinant [23–25], i.e., it determines interaction of M8 with proteins such as Ato and Sens. Moreover, it has been reported that two motifs of the Orange domain, EVS and THL (Fig. 4a) are necessary for the M8-Sens interaction [24]. In their analysis, the authors found that replacement of both motifs with Ala residues abrogated interaction with Sens. The possibility that these motifs are specificity-determinants for Sens remained perplexing, because they are invariant in six out of seven *Drosophila* E(spl) members (Fig. 4a and not shown). Moreover, the Glu and His residues of both motifs are highly conserved in mammalian and amphibian proteins of the HES/HEY family (data not shown), and in the case of *Xenopus* BOIP [44] Orange appears to serve as an extended dimerization domain. More recently, Wong and co-workers (RIKEN Structural Genomics/Proteomics Initiative) reported the structure of the Orange domain of human HESR1 (PDB file 2DB7, unpublished), which shares significant similarity to the Orange domain of *Drosophila* M8. In their structure, the Orange domain is a dimer, wherein the EVS motif of one member appears proximal to the THL motif of the second member in a conformation that resembles a 'double hairpin' (Fig. 4c).

We thus tested variants of M8 where the EVS and/or THL motifs were replaced by Ala residues. Surprisingly, these variants do not lack dimerization capacity, but instead form dimers with a strength that is approximately 4-fold higher than that of wild type M8 (Fig. 4c). Given that a number of hydrophobic residues flank the EVS/THL motifs (Fig. 4b), it appears more likely that the Ala mutations engender excessive stabilization of the dimer of the Orange domain via hydrophobic interactions, and their high conservation through evolution would argue that these are structural, rather than specificity, determinants.

The invariant Gly residue in Orange is critical for dimer formation

The Orange domain of *Drosophila* M8, like HESR1, contains a Gly-Tyr pair, N-terminal to the EVS motif (Fig. 4a). In the extended family of invertebrate and vertebrate E(spl)/HES/HEY bHLH proteins, the corresponding residues are Gly-Tyr or Gly-Phe; with Gly being invariant throughout evolution (Fig. 4B and not shown). In addition, this Gly residue is located at an interface where the two monomers make asymmetric contact (Fig. 5a). The juxtaposition of two Orange monomers could best be achieved with a Gly residue, and it is

likely that its replacement with any other residue would displace the two monomers, perturb the interaction of the EVS-THL motifs and disrupt dimer formation.

A revertant allele of *E(spl)D* replaces the invariant Gly residue of the Orange domain

As stated above (see Introduction), the unique dominant *E(spl)D* mutation, results in a mutant protein, M8*, that is independent of CtD-dependent auto-inhibition. Since the identification of this allele in 1956 [45], follow-up studies from a number of labs have sought to better understand why this mutant protein perturbs eye development [25, 33, 46, 47].

During formation of the *Drosophila* eye, Ato is essential for the specification of the founding R8 photoreceptor [48–50]. The expression of Ato in the PNC's is resolved by E(spl)-M8 [51, 52], leading to the specification of a single R8 from each PNC. Each R8 cell then recruits surrounding uncommitted cells (reviewed in [4, 53–55]), driving formation of the highly ordered adult eye in which each hexagonal facet (unit eye) contains 8 photoreceptors, pigment and cone cells. It has also been shown that *E(spl)D* (M8*) exhibits excessive binding to Ato, resulting in a loss of the R8 cells [25, 47]. As a result, no other retinal cell types are specified, leading to loss of virtually the entire eye field (Fig. 5).

A revertant of *E(spl)D* was isolated and molecularly defined by the lab of Anette Preiss [25]. This revertant, called *E(spl)D^{revBE22}* (Fig. 5c), harbors a Gly⁸⁶Asp mutation, predicted to lie at the interface of the Orange dimer (Fig. 5a). In the molecular model, side chain collisions and electrostatic repulsion of two vicinal Asp residues is predicted to prevent juxtaposition of the two monomers, and consequently impair interaction of the EVS and THL motifs as well (Fig. 5a). The possibility arose that the *E(spl)D^{revBE22}* revertant allele replaces a key structural determinant for Orange dimerization. If so, a variant of M8 with a Gly⁸⁶Asp replacement should be impaired for dimerization, and our studies indicate this to, indeed, be the case (Fig. 5c). It therefore appears that the loss of function (revertant) behavior of *E(spl)D^{revBE22}* reflects loss of dimer formation. Because the eye defects of M8* appear to primarily reflect its binding to Ato, we propose that proper dimer formation is a pre-condition for interaction with and antagonism of Ato.

Implications of findings to repression by E(spl) proteins

To our knowledge, this is the first indication that the Orange domain of M8 is an extended dimerization domain, further extending its similarity to *Xenopus* BOIP [44]. Our studies raise the likelihood that this property might be more common to the bHLH repressors of the E(spl)/HES/HEY family than has been previously recognized. In the case of M8, our studies resolve some long-standing issues on the mechanism of repression, and raise new questions.

Dimer formation

In the case of E(spl) proteins, dimer formation has been thought to primarily involve the first HLH domain. Rather, our studies indicate that Orange contributes significantly to dimer formation. The intermolecular nature of this interaction in the case of the Orange domain raises the possibility that this may, perhaps, occur first, with the HLH domain acting subsequently. This model would be consistent with our observation that, compared to M8*, the M8*-G86D variant is devoid of dimerization capacity, rather than simply interacting with reduced strength. The exacerbated dimerization capacity of M8 harboring Ala substitutions at the EVS and THL motifs (Fig. 4), together with its inability to interact with Sens and mediate repression [24], are of particular interest, because they suggest that a stabilized Orange dimer is non-permissive for repression. If so, the question arises whether the inter-Orange interactions (the 'double hairpin') might need to be remodeled to permit binding to Ato and Sens, as part of the repression mechanism. Another question raised by

these studies is on autoinhibition by the CtD, and whether this domain stabilizes the 'double hairpin' to restrain repression, and if so, how.

Gro recruitment

Our studies on Gro binding raise questions on the reported sufficiency of WRPW for this crucial interaction [28]. While deletion of this motif, or its replacement by WLPW (our unpublished data) abolishes the M8-Gro interaction, this motif is not context-independent. Our studies show that WRPW is insufficient for Gro recruitment when positioned either adjacent to the phosphorylation domain (Fig. 2), or adjacent to the Orange domain (data not shown). The inability of the latter construct (M8*-Gro, see Fig. 1b) is of particular relevance, because the activity of just such a construct has been tested *in vivo*. In this study, Giebel and Campos-Ortega [33] reported that *in vivo* expression of M8*-Gro did not elicit any reduced eye in the presence of wild type Notch activity. As M8* cannot recruit Gro, it requires a sensitized *N^{spl}* background. It would thus appear that the presence of a WRPW motif is insufficient to *a priori* predict Gro-binding capacity. While the structural reasons for this insufficiency are presently unclear, we speculate that the 56 residues that comprise the CtD may provide optimum positioning of the WRPW motif and thereby influence Gro binding. In this context, it is noteworthy that Gro forms tetramers, a conformation that appears to be necessary for repression *in vivo* [56, 57]. The possibility thus arises that in the M8 dimer, the CtD may position the WRPW motif at an optimal distance from the Orange 'double hairpin' to enable recruitment of Gro tetramers. Given the role of the CtD in autoinhibition, another question that arises is whether the 'autoinhibited' state of M8 is refractory to Gro binding. If so, it will suggest that it is the CtD that coordinates repression.

In summary our studies provide new structural insights into M8, a paradigm for neural repression. They indicate that inter-domain interactions and dimer formation are important in regulating repression. Future studies to determine the three dimensional structure of this protein will be necessary to more precisely parse the structural alterations induced by phosphorylation, and their effects on dimerization, Gro-recruitment, and repression.

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Fig. 1. Regulation of M8 activity by phosphorylation and structure of variants

a Model for auto-inhibition of M8 and its regulation by CK2. In the off state, the C-terminal 56 residues (CtD) interacts with either the HLH or Orange, thereby blocking dimer formation and/or interaction with proneural activators. Phosphorylation by CK2 displaces the CtD to permit formation of homo/hetero-dimers and Gro binding. **b** Schematic of variants used in the studies. The full-length protein is shown with the relative positions of known functional domains. Internal deletions are denoted as dotted lines

Fig. 2. Interaction of M8 variants

Plasmids expressing M8-variants as fusion with the DNA-binding domain (BD) of Gal4 were tested against fusions of CK2 α , CK2 β , or Gro with the activation domain (AD) of Gal4 in yeast strains pJ69-4a and pJ69-4 α . Diploids were plated onto complete (Ade⁺) or minimal media lacking adenine (Ade⁻) and incubated at 29°C for 3–4 days

Fig. 3. Interaction of M8 variants with Atonal

Yeast strain EGY048 was transformed with plasmids expressing M8-variants as fusions with the DNA-binding domain of LexA in combination with either the AD alone or the AD-Atonal fusion protein. Transformants were grown in galactose/raffinose media, and *LacZ* activity (Miller units) was determined in triplicate for three-six independent transformants

Fig. 4. A model of the structure of the Orange domain, and its role in dimer formation
a Alignment of the Orange domain of *Drosophila* (d) M8, M7, M5 and human (h) Hes6. Residues conserved in the Orange domain are highlighted. **b** Model of the Orange domain of M8, based on the structure of the Orange domain of human HESR1 (PDB file 2DB7). **c** Dimerization assay of M8 variants with Ala residues in place of the EVS and TLH motifs. Interactions were assayed in the LexA-based two-hybrid assay, and *LacZ* activity (Miller units) was determined in triplicate for three-six independent transformants

Fig. 5. A highly conserved glycine residue is critical for Orange dimerization

a The Gly residue localizes at the interface of the Orange dimer, and its replacement by Asp is likely to interfere with the juxtaposition of the first major helix. **b** Eye defects of the *m8* mutation *E(spl)D*. Note that *E(spl)D* only elicits a reduced eye in the presence of the *Notch* allele, *N^{sp}*. **c** Dimerization assay of M8* encoded by *E(spl)D*, and a variant M8*-G⁸⁶D encoded by *E(spl)D^{revBE22}* that has lost its ability to elicit a reduced eye.