Drosophila melanogaster Casein Kinase II Interacts with and Phosphorylates the Basic Helix-Loop-Helix Proteins m5, m7, and m8 Derived from the Enhancer of split Complex

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Regina L. Trott, Madhavi Kalive, Zeev Paroush‡, and Ashok P. Bidwai§

From the Department of Biology, West Virginia University, Morgantown, West Virginia 26506-6057 and the §Department of Biochemistry, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

Casein kinase II (CKII) is a ubiquitous protein kinase that is highly conserved among eukaryotes (1, 2) and is capable of functioning as an oncogene in mammals (3). CKII is composed of catalytic (α) and regulatory (β) subunits that combine to form an αβ2 holoenzyme. With the exceptions of Drosophila melanogaster (4), Caenorhabditis elegans (5), and Schizosaccharomyces pombe (6), CKII from most eukaryotic organisms contains two α subunits, α1 and α2, that are encoded by distinct genes. In contrast, β subunit heterogeneity has been documented via protein microchemical approaches in Saccharomyces cerevisiae (7) and via molecular/genetic approaches in Arabidopsis thaliana (8) and D. melanogaster (9).

CKII preferentially phosphorylates Ser/Thr residues in an hyperacidic context (10), although phosphorylation of Tyr has been documented in at least one case, i.e. yeast Fpr3 (11). Analysis of the phosphorylation of synthetic peptides suggests that the consensus site for phosphorylation by CKII can best be described as (S/T)(E/D)X(D/E) (10). Consistent with this, a number of proteins critical for transcription, cell cycle regulation, and signal transduction contain such a site(s) and are known to be phosphorylated in vitro and in vivo (12). Although CKII activity is inhibited in vitro by polyacidic compounds, such as polyaspartate and polyglutamate (13), and stimulated by polybasic compounds, such as polylysine and protamine (14), the in vivo relevance of these observations is currently unknown. Comparisons between recombinant monomeric α subunit and native or reconstituted αβ2 holoenzyme have revealed that the β subunit plays a complex role in regulating the basal activity of the α subunit (15–17). Although the β subunit stimulates the activity of the monomeric α subunit 5-fold against most substrates, it down-regulates phosphorylation of a select few proteins, notably calmodulin (14, 18), the actin bundling protein, Sac6p,2 and a novel Drosophila zinc finger protein, ZFP35.3 The β subunit is also subject to phosphorylation by the α subunit (4), but the biological role of this reaction remains undefined.

Genetic analyses in budding and fission yeast have demonstrated that the enzyme is essential for viability (19, 20). Studies utilizing temperature-sensitive alleles of the α subunits of yeast CKII indicate a requirement of the enzyme for cell cycle progression in G1 and G2/M (21), in the maintenance of cytoskeletal architecture (22), and for cytokinesis (20). In contrast to the two yeast models, analysis of DmCKII has been stymied, principally due to the absence of mutations, even

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‡ To whom correspondence should be addressed: Dept. of Biology, P. O. Box 6057, Brooks Hall, West Virginia University, Morgantown, WV 26506-6057. Tel.: 304-293-5201 ext. 2533; Fax: 304-293-6363; E-mail: Abidwai@wvu.edu.

1 The abbreviations used are: CKII, casein kinase II; HLH, helix-loop-helix; bHLH, basic HLH; E(sp1), Enhancer of split; E(sp1)/C, Enhancer of split complex; Dm, D. melanogaster; DB, DNA binding; AD, activation domain; GST, glutathione S-transferase; SDS, sodium dodecyl sulfate.

2 A. P. Bidwai, unpublished data.

3 M. Kalive, R. L. Trott, and A. P. Bidwai, unpublished data.
though the cDNAs encoding DmCKII were the first to be isolated (23). However, two complementary approaches that have recently been applied to DmCKII have proven to be exceptionally useful for analyzing functions of this kinase in a metazoan context. The first of these is the two-hybrid system (24) that has been used to identify CKII interacting proteins, many of which appear to be potential substrates of this kinase. The second is genetic analysis on these interacting proteins via targeted misexpression of transgenes encoding nonphosphorylatable and constitutively phosphorylated variants using the Gal4-UAS system (25), followed by phenotypic analysis. Studies along these lines have identified a novel regulatory β′ subunit of DmCKII (9), suggested the presence of five alternative transcription start sites in the CKIIβ gene (26), and demonstrated an interaction of the homebox protein Antennapedia, ANTIP, with DmCKIIa (27). In the case of ANTIP, phosphorylation by DmCKII appears necessary for restricting its activity during embryogenesis. A somewhat similar situation exists for another homeobox protein, Engrailed, which also appears to be regulated by CKII-mediated phosphorylation (28). In addition, the serine proteinase Dishevelled, a component of the wingless/Wnt signaling pathway (29), is a target for CKII, and both proteins exist as a complex in vivo (30). Collectively, these results suggest that CKII plays crucial roles in embryonic development as well as in cellular differentiation.

In an attempt to better define the physiological role of CKII, we have used the two-hybrid approach (24) to identify and characterize physiological partners of the β′ subunit of DmCKII (DmCKIIβ). One of the proteins identified in this screen is m7, a bHLH-type transcription factor derived from the neurogenic locus E(spl)c (31). The E(spl)c encodes the structurally and functionally similar bHLH proteins mC (also known as mlocus) and mA (also known as m8), mA (also known as m3, m5, m7, and m8 (32, 33) and is epistatic to other neurogenic loci, such as Notch, Delta, etc. (34–36). The segregation of neural and eidermal lineages during development is determined by cell-cell communications that involve two interacting sets of genes: the neurogenic genes mediate signals between adjacent cells, and the proneural genes promote neural development. This nomenclature is explained by the fact that the neurogenic genes are named for their loss-of-function phenotype, whereas the proneural genes are named for their normal function. In a process termed “lateral inhibition,” Delta provides the inhibitory signal, which encodes the bHLH protein m7, is the subject of this study, whereas other wills be reported elsewhere.

Explicit interactions between DmCKIIa and E(spl) proteins were studied in the LexA-based version of the two-hybrid system (49) that was developed in the laboratory of Roger Brent (henceforth referred to as the Brent system). In the Brent system, proteins to be tested for interaction are expressed as fusions with the DNA binding (DB) domain, and activation domain (AD) of a bHLH protein mC. Using site-directed mutagenesis in an AD

**EXPERIMENTAL PROCEDURES**

**Construction of Two-hybrid Plasmids—** DNA corresponding to amino acids 1–356 of DmCKIIa and amino acids 1–215 of DmCKIIβ was amplified by polymerase chain reaction using primers containing two terminal 5′ bases, a restriction site, and 20 bases of exact homology to the start and stop codon regions. The polymerase chain reaction products were subcloned into the plasmids pGBT9 and pGAD424 (gift of S. Fields, University of Washington) and completely sequenced using the Prism Dye Terminator Cycle sequencing kit (Applied Biosystems). The resulting plasmids express DmCKIIa and DmCKIIβ as C-terminal fusion with the DNA binding (DB) domain, and activation domain (AD) of S. cerevisiae Gal4 (44), respectively.

**Gal4-based Yeast Two-hybrid Screening—** Screens were conducted in the Gal4-based version of the two-hybrid system (henceforth referred to as the Yeast system) using the yeast strain HFTC (MATα, ura3–52, his3–200, leu2–3, 112, trp1–201, 112, gal4–538, lys2–582, GAL1–GAL1p-HIS3, URA3–GAL4BD-cell-CYC1::GAL4DB-DmCKII (50). HFTC expressing Gal4DB-DmCKIIa (the bait) was used to screen a 3–18 h D. melanogaster embryo two-hybrid cDNA library (gift of S. J. Elledge, Baylor College of Medicine). This library is contained in the plasmid pACT, which expresses cDNA-derived proteins as C-terminal fusions with Gal4AD (46). A total of 2 × 10⁵ transformants were plated on glucose dropout medium lacking tryptophan, leucine, and histidine (47), and colonies exhibiting rapid growth were counted for expression of LacZ (48). Of the 45 His + colonies, 15 tested positive for LacZ and were therefore chosen for further analysis. The library plasmids containing the yeast LEU2 gene were selectively recovered via complementation of the leucine auxotrophy of Escherichia coli HB101 (47). The isolated plasmids were subsequently used to retransform HFTC expressing Gal4DB-alone, GALAD-DmCKIIa and GALAD-DmCKIIβ. These cDNAs that induced expression of HIS3 and LacZ only in response to GALAD-DmCKIIa, i.e., a bait-specific manner, were identified by sequencing their 5′- and 3′-ends using the primers 5′-ATACACTACAATGATTGATGATC-3′ and 5′-ACAGTTGAGATTGAAATTGGC-3′, respectively. All novel cDNAs were completely sequenced using custom primers as described above. One of these cDNAs, DmAS1, which encodes the bHLH protein m7, was the subject of this study, whereas others will be reported elsewhere.

**LexA-based Two-hybrid Interactions—** LexA-based Two-hybrid Interactions—LexA-based version of the two-hybrid system (49) that was developed in the laboratory of Roger Brent (henceforth referred to as the Brent system). In the Brent system, proteins to be tested for interaction are expressed as fusions with the DNA binding domain of the bacterial repressor, LexA, and the activation domain of protein B42. The yeast strain used for these studies was EGY048 (MATa, trp1, his3, ura3, leu2), which harbors a single chromosomally integrated copy of the yeast LEU2 gene under the control of six LexA operators, and a high copy plasmid, pSH18-34, which expresses E. coli LacZ under the control of eight LexA operators (50). Therefore, expression of the two reporter genes, LEU2 and LacZ, is induced when the interacting complex is tethered to the LexA-operators. Additionally, expression of the AD fusion protein is under control of a GAL-promoter. As a result, reporter gene expression, in an AD fusion protein-dependent manner, is only observed when cells are grown in media containing galactose, but not glucose, as the sole carbon source. Yeast EGY048 containing plasmid pSH18-34 was transformed with a plasmid expressing the B42-derived AD-alone (49) or AD-DmCKIIa fusion protein using lithium acetate (47). A single transformant was selected and subsequently retransformed with plasmids expressing LexA-m7, LexA-m8, LexA-m5, LexA-m3, and LexA-mC (38). Three independent transformants were tested for induction of the LEU2 gene on glucose- and galactose-dropout medium lacking leucine (47) at 29 °C for 4 days. In parallel, cultures were analyzed in triplicate for Gal4-dependent transformants were tested for induction of the LEU2 gene were selectively recovered via complementation of the leucine auxotrophy of Escherichia coli HB101 (47). The isolated plasmids were subsequently used to retransform HFTC expressing Gal4DB-alone, GALAD-DmCKIIa and GALAD-DmCKIIβ. These cDNAs that induced expression of HIS3 and LacZ only in response to GALAD-DmCKIIa, i.e., a bait-specific manner, were identified by sequencing their 5′- and 3′-ends using the primers 5′-ATACACTACAATGATTGATGATC-3′ and 5′-ACAGTTGAGATTGAAATTGGC-3′, respectively. All novel cDNAs were completely sequenced using custom primers as described above. One of these cDNAs, DmAS1, which encodes the bHLH protein m7, was the subject of this study, whereas others will be reported elsewhere.

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mid 

**RESULTS AND DISCUSSION**

**Isolation of cDNAs Encoding m7**—The yeast strain HF7C expressing Gal4DB-DmCKIIa as a bait was used to screen a D. melanogaster embryo two-hybrid cDNA library. From $2 \times 10^6$ transformants, 15 clones that activated transcription of HIS3 and LacZ were recovered. All 15 clones induced the two reporter genes only when cotransformed with DmCKIIa (data not shown). Sequencing of the cDNAs revealed that seven of the clones encode DmCKIIβ (26), one encodes DmCKIIa (9), two encode DmCKIIβ (a novel isoform of the β subunit (9)), one (DmA51) encodes m7, and the rest encode novel proteins that will be described elsewhere. The library plasmid was recovered from yeast clone DmA51 and restested for interaction against various bait constructs. As shown in Fig. 1A, induction of HIS3 and LacZ was observed only when yeast HF7C coexpressed Gal4AD-m7 with Gal4DB-DmCKIIa. On the other hand, neither reporter gene was induced when HF7C was transformed with Gal4AD-m7 by itself or in combination with a plasmid encoding either Gal4DB-alone or Gal4DB-DmCKIIβ, suggesting that m7 interacts specifically with the catalytic subunit of DmCKII. The inability of DmCKIIβ to interact with m7 is not due to a lack of expression of the former protein because this construct is expressed in yeast and displays a strong interaction with DmCKIIa (9).

Sequencing revealed that the DmA51 cDNA encodes full-length m7 and contains 78 and 228 base pairs of sequence, 5′ to the initiation codon (ATG) and 3′ to the termination codon (TAA), respectively. This cDNA, which does not contain any in-frame stop codons 5′ (TAA), respectively. This cDNA, which does not contain any in-frame stop codons 5′ to the initiation codon, is identical to base pairs 172–1068 of a 4.4-kilobase genomic clone (Fig. 1B) that encodes the m7 and m8 transcription units, each on a single uninterrupted exon (55). The absence of a poly(A) tail in clone DmA51, combined with the presence of a single poly(A) addition signal in the corresponding gene at position 1250 (Fig. 1B), suggests that the isolated cDNA is not full-length with respect to its 3′ untranslated region. In this regard, we have recently rescreened the Drosophila cDNA library for DmCKIIa-interacting proteins and have isolated two additional clones, DmA002 and DmA130, that also encode m7. Our isolation of multiple cDNAs encoding m7 from two independent two-hybrid screens strengthens the likelihood of the relevance of its interaction with DmCKIIa. Apart from length heterogeneity with respect to the DmA51 cDNA, the DmA002 and DmA130 sequences are identical to the corresponding region of the m7 transcription unit and display no polymorphisms (data not shown).

**Interaction of DmCKIIa with E(spl)-derived bHLH Proteins**—The observed interaction between DmCKIIa and m7 was surprising, as there was no previous indication that m7 is regulated by phosphorylation or that CKII is involved in neurogenesis. Given the structural similarity of all E(spl) proteins (32), we were interested in determining whether DmCKIIa also interacts with other members derived from this locus. For this

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**Fig. 1A**

**Fig. 1B**
activity observed for mC in combination with the AD-alone and its “silencing” upon expression of AD-DMcCKIIa are consistent with our observation that the unfused AD, in a limited number of cases, confers basal transcription of the reporter genes that is abolished upon expression as a fusion protein.\(^4\) That DMcCKIIa-m5, -m7, and -m8 interactions display a higher \(\gamma\) activity (Fig. 2) than does DMcCKIIa-DMcCKIIb (Fig. 1A) does not imply that the former protein pairs interact with a higher affinity. As outlined under “Experimental Procedures,” this is a reflection of the high copy/affinity \(\beta\) expression of a fusion protein reported. In addition, no induction of \(\gamma\) LacZ activity was observed when transformants were grown in rich glucose medium (data not shown), suggesting that reporter gene expression was dependent on presence of the AD-DMcCKIIa fusion protein. Identical results were observed for the second reporter gene, \(\delta\) LacZ (data not shown). It should be noted that the two-hybrid interaction between DMcCKIIa and m7 was originally detected using the former protein as a Gal4DB fusion and the latter as a Gal4AD fusion (Fig. 1A), whereas the explicit testing involved the inverse orientation, i.e. m7 as a LexA fusion and DMcCKIIa as an AD fusion (Fig. 2). These results demonstrate that the interactions are neither orientation-specific, as is the case of the interaction of DMcCKIIa with DMcCKIIb (9), nor dependent on a specific version of the two-hybrid system.

The apparent specificity of DMcCKIIa for m5, m7, and m8 but not for other members tested (m3 and mC) was surprising given that these proteins are structurally conserved (32). Of particular note are two motifs, the highly conserved HLH domain (32), which mediates interaction with Groucho (38). We found, however, that variants of m7 lacking either of these motifs interact as effectively with DMcCKIIa (9), as does the wild-type protein (data not shown). These results are consistent with our inability to detect interactions of DMcCKIIa with m3 and mC (both of which contain the HLH and WRPW motifs) and suggest that the interaction domain lies elsewhere in m5/7/8.

**Conservation of a CKII Site in m5, m7, and m8**—As men-
Phosphorylation of m5, m7, and m8 by Drosophila CKII—One question raised by the sequence alignment was whether the presence of the consensus CKII site in m5, m7, and m8 correlates with their phosphorylation. We have therefore subjected GST-alone, GST-m5, GST-m7, GST-m8, and GST-mC, a non-interacting member, to phosphorylation using two isofoms of CKII, i.e., the monomeric α subunit purified from a yeast expression system (15), and the αβ2 holoenzyme purified from embryos (4). The former isoform mimics the two-hybrid analysis (Fig. 2), whereas the latter mimics the in vivo environment. The results demonstrate that m5, m7, and m8 are phosphorylated by both isoforms of CKII (Fig. 4, B and E, lanes 2–4) and corroborate their observed two-hybrid interaction with DmCKIIα. No phosphorylation of either GST or GST-mC (Fig. 4, B and E, lanes 1 and 5) was observed with either enzyme isoform, demonstrating the absence of phosphorylation of the affinity tag used for purification and suggesting that phosphorylation is specific only to those E(spl) proteins that also exhibit a two-hybrid interaction with DmCKIIα. At a quantitative level, however, the rates of phosphorylation of the three E(spl) proteins are different for both enzyme isoforms, such that m5 > m7 = m8 (compare lanes 2, 3, and 4 in Fig. 4, B and E). What mechanism can account for the observed differences? Detailed kinetic analysis of CKII suggests that whereas DmCKIIα and the holoenzyme display virtually identical $K_m$ values for the protein substrate, the $K_{cat}$ can differ 5–50-fold in a substrate-dependent manner (14). Furthermore, studies with peptides suggest that whereas the acidic residues at n+1 and n+3 are absolutely required for phosphorylation, additional acidic residues C-terminal to the n+3 position further increase the $K_{cat}$ with marginal effects on the $K_m$ (57, 61). These criteria, therefore, make it possible to predict the relative rates of phosphorylation of m5/7/8. In this regard, although m7 and m8 fit the consensus, m5 is probably the best because it contains an additional Asp at the n+4 position (Fig. 3B). The rank order for phosphorylation is, therefore, predicted to be m5 > m7 = m8. The analysis presented here essentially reflects this prediction. Because the gel analysis described here inherently reflects a semiquantitative assessment of phosphorylation, kinetic analysis will be necessary to determine whether the observed differences in phosphorylation of m5 versus m7/8 are due to differing catalytic efficiencies ($K_{cat} / K_m$). That CKII interacts with and phosphorylates these proteins is consistent with the observation that this kinase has been found to exist in a complex with some of its in vivo substrates, such as Topoisomerase II (62), HSP90 (63), ANTP (27), and Dishevelled (30), to name a few.

We and others have previously demonstrated that polybasic compounds, e.g., polylsine, overcome a down-regulation of the holoenzyme that can be conferred by the β subunit of CKII for some substrates (14, 64). We were therefore interested in determining whether the limited phosphorylation of m7 and m8, relative to that of m5, might be sensitive to polylsine addition. The results suggest that phosphorylation of m5 is virtually unaffected by this compound when tested with either DmCKIIα (compare Fig. 4B, lane 2, to Fig. 4C, lane 2) or DmCKII holoenzyme (compare Fig. 4E, lane 2, to Fig. 4F, lane 2). On the other hand, phosphorylation of both m7 and m8 is stimulated by polylsine addition (compare lanes 3 and 4 in Fig. 4B versus 4C and 4E versus 4F). These results are consistent with previous
Drosophila CKII Phosphorylates m5, m7, and m8

**Fig. 4.** Phosphorylation of m5, m7, and m8 by DmCKII. The indicated GST fusion proteins were purified and subjected to phosphorylation using the monomeric α subunit (DmCKIIα) and the αβ subunit (DmCKIIa) of Drosophila embryos. A representative gel stained with Coomassie Blue shows the amount and purity of the various GST fusion proteins that were phosphorylated with either DmCKIIα (A) or the holoenzyme (D). Proteins were phosphorylated in either the absence (B and E) or in the presence (C and F) of 100 μg/ml poly(L-lysine). Samples were electrophoresed in 12% SDS-polyacrylamide gels, stained with Coomassie Blue, and autoradiographed (B, C, E, and F). Arrows in A and D indicate the mobilities of the full-length fusion proteins.

**Fig. 5.** Interaction of DmCKII with m8. Bacterially expressed GST fusion proteins immobilized on glutathione-Sepharose beads were incubated with Drosophila embryo holoenzyme. The beads were separated from the unbound material as described under "Experimental Procedures," and the bead-bound (P, pellet) and the unbound (S, supernatant) samples were examined by Western blotting using antibodies that specifically recognize the m8 protein (68).

**Analysis:**

The full-length fusion proteins were purified and subjected to phosphorylation using the monomeric α subunit (DmCKIIα) and the αβ subunit (DmCKIIa) of Drosophila embryos. A representative gel stained with Coomassie Blue shows the amount and purity of the various GST fusion proteins that were phosphorylated with either DmCKIIα or the holoenzyme. Proteins were phosphorylated either in the absence (B and E) or in the presence (C and F) of 100 μg/ml poly(L-lysine). Samples were electrophoresed in 12% SDS-polyacrylamide gels, stained with Coomassie Blue, and autoradiographed (B, C, E, and F). Arrows in A and D indicate the mobilities of the full-length fusion proteins.

**Significance of Results:**

Phosphorylation analysis indicating that phosphorylation of substrates with optimal sites (such as the RII subunit of cAMP-dependent protein kinase) responds modestly, if at all, to polylysine, whereas those that satisfy the minimal requirements of CKII (such as calmodulin) are more responsive (14). This stimulation by polylysine is not a reflection of promiscuous phosphorylation, because DmCKIIα or the holoenzyme do not phosphorylate GST or GST-mC (lanes 1 and 5 in Fig. 4, C and F) in the presence of polylysine. However, no cellular protein that can mimic the "polylysine effect" with respect to CKII has so far been identified, unlike the case with Ras, which mediates the polylysine-dependent phosphorylation of calmodulin by the insulin-receptor kinase (65).

Is there any evidence, apart from our two-hybrid and phosphorylation analysis, to suggest that m5/7/8 are more closely related to each other than are other E(spl) proteins? We believe that molecular/genetic analyses do, in fact, support this proposal. Using a bacteriophage λ-based system to detect protein-protein interactions, Gigliani et al. (43) suggest that m5, m7, and m8 are the most closely related. Although yeast two-hybrid analysis conducted by Alifragis et al. (42) essentially reiterates the closest similarity between m8 and m5, they suggest, however, that m7 be clustered along with mA and mB, a proposal at odds with their own genetic analysis (see below). Because E(spl) proteins homo/heterodimerize and interact with proneural proteins as well, *in vivo* associations between these proteins is needed to clarify the differences, if any, with regards to m7. Furthermore, and perhaps the most persuasive, is genetic analysis demonstrating that the severity of suppression of bristle development, *i.e.* neurogenesis, closely correlates with ectopic expression of only m7 and m8 (66). A similar analysis with m5 was, however, precluded even with two copies of the transgene, leading the authors to conclude that, of the seven E(spl) proteins, m5 is probably most inactive/unstable (66). Northern or Western analyses of the m5 and m6 transgenes will be necessary to clarify whether this is indeed the case. Given the functional equivalence of m5/7/8 in neurogenesis, we were specifically interested in determining the mechanism by which these proteins interact with DmCKII. We have deferred conducting parallel, and thus redundant, analysis on all three proteins and have selected m8 for these additional studies. This choice was based on our eventual goal of analyzing the significance of this interaction in transgenic flies and to remain consistent with genetic analysis on this protein (see below and Ref. 67) via the GAL4-UAS system (25).

**Interaction of m8 with DmCKII**—Although the available data demonstrate that m5/7/8 interact with DmCKII and are phosphorylated by it, they do not indicate whether these proteins are capable of direct physical association. Analysis of complex formation between these proteins in the developing *Drosophila* embryo is currently precluded by the absence of antibodies that specifically recognize the m8 protein (68), coupled with its restricted expression domains within the neuroectoderm (33). As an alternative, we have assessed the ability of recombinant bacterially expressed GST-m8 to form a physical complex with CKII purified from *Drosophila* embryos. To this end, GST-alone and GST-m8 were purified, immobilized on glutathione-Sepharose beads, and tested for their ability to form a complex with *Drosophila* embryo CKII.

The presence of
DmCKII in the bead-bound (pellet) and unbound (supernatant) fractions was assessed by Western blotting using an antisera that recognizes both subunits (α and β) of DmCKII (54). As expected, incubation of the GST beads with DmCKII did not result in any immunoreactive material in the pellet fraction, indicating the absence of an interaction (Fig. 5, compare lanes P and S). On the other hand, incubation of GST-m8 beads with DmCKII resulted in the presence of immunoreactive material in the pellet fraction (Fig. 5, compare lanes P and S), demonstrating that these two proteins form a physical complex. These results suggest that the two-hybrid interaction of DmCKII with m8 is direct and is unlikely to be mediated by the recruitment of yeast proteins.

Mapping the Site of Phosphorylation on m8—We next sought to define the site of phosphorylation. We therefore generated two variants of m8 with substitutions of the conserved Ser in the CKII site, i.e. m8S159A and m8S159D. The former is a nonphosphorylatable variant, whereas the latter should mimic the constitutively phosphorylated protein, in line with studies on ANTP (27), HP1 (69), etc. GST-m8, GST-m8S159A, and GST-m8S159D fusion proteins were purified and subjected to phosphorylation using DmCKII and the holoenzyme. The results demonstrate that GST-m8 is phosphorylated by the holoenzyme and the α subunit (Fig. 6B, lanes 1 and 4). On the other hand, neither GST-m8S159A (Fig. 6B, lanes 2 and 5) nor GST-m8S159D (Fig. 6B, lanes 3 and 6) are substrates of the two enzyme isoforms. This result strongly suggests that CKII phosphorylation of m8 occurs at Ser159, and by corollary the site of phosphorylation on m7 and m5 is most likely to be Ser168 and Ser156, respectively (see Fig. 3B). We consider it unlikely that GST-m8S159A and GST-m8S159D are partially clipped leading to abolished phosphorylation, because, relative to GST-m8, neither protein exhibits altered mobility in SDS-polyacrylamide gels (Fig. 6A, compare lane 1 with lanes 2 and 3). In addition, the inability of the two variants to be phosphorylated by CKII suggests that m8 contains a single site for phosphorylation by CKII, thus corroborating our sequence-based prediction (see Fig. 3B). The ability of m8 to be phosphorylated by DmCKII is, however, dependent on the holoenzyme at the identical residue is also consistent with our contention that the substrate specificity of this enzyme is intrinsic to the α subunit (14).

Interaction of m8S159A and m8S159D with DmCKII—We were interested in determining whether phosphorylation of m8 affects its interaction with CKII. We, therefore, determined the interaction of DmCKIIs with m8S159A and m8S159D, relative to wild-type m8. As shown in Fig. 7, replacement of Ser159 with Ala decreased interaction by −50%, whereas replacement with Asp abolished the interaction. These results suggest that the interaction of DmCKIIa with m8 appears analogous to that of an enzyme with its substrate and is in line with the interactions of the protein kinase, Snf1, with its substrate, Snf4 (70). Our interpretation of the results is, however, complicated by the fact that phosphorylation of m8 appears to disrupt the complex and may affect protein stability as well (see below). The former possibility is likely, given that DmCKIIa is catalytically active when expressed in yeast (15), and suggests that the strength of the two-hybrid interaction observed between DmCKIIa and m5/7/8 may, in fact, represent an underestimate. The likelihood of the latter possibility is difficult to predict, at least in the context of the yeast system used in this study, given that two-hybrid interaction of m8S159D with Groucho appears identical to that observed for wild-type m8.4 These results suggest that accessory proteins, perhaps lacking in yeast, may be necessary for affecting stability of m8S159D in Drosophila. In addition, although D. melanogaster m5/7/8 and D. hydei m8 contain the conserved sequence, (L/I)SP(V/I)A/SSGY, flanking the phosphorylation site (see Fig. 3B), it is presently unknown whether these residues contribute binding energy in addition to that attributable to the CKII site. Studies with Snf1/Snf4 have, in fact, demonstrated the involvement of flanking residues in mediating interactions (71). Further analysis will be needed to determine the intrinsic affinities (Kd) of these bHLH proteins for DmCKII, rather than those (km) inferred by kinetic analysis.

Implications of Phosphorylation of m5, m7, and m8—The results presented above raise the likely prospect that DmCKII interacts with m5/7/8 when these proteins are in the nonphosphorylated state and that the complexes dissociate upon phosphorylation. We obviously cannot extrapolate the two-hybrid and biochemical results to the situation in the epidermal precursors in the developing Drosophila embryo with certainty. However, given the requirements of CKII for cell cycle progression (21) and for checkpoint control (72), it is likely that epidermal progenitors, which are expressing E(spl) proteins, which are expressing E(spl) proteins, also contain CKII. A direct test of this proposal in the developing embryo is still remains a difficult task due to restricted expression of m5/7/8 and the absence of isoform-specific antibodies (see above). At a functional level, our data indicate that interaction and/or phosphorylation of m5/7/8 is unlikely to affect their DNA binding properties (which require the basic region), their ability to heterodimerize with proneural proteins (which requires the HLH domain), or their ability to interact with Groucho (which requires the WRPW motif). What function could then be ascribed to interaction and/or phosphorylation?
that of NF-A mechanistically similar situation appears to regulate within a motif with a high PEST score (74) and undergoes embryo involves the activities of a transcription factor, sopherila and by extension those in The structural and functional properties common to m5/7/8, m5/7/8 may influence the stability of these proteins tations are consistent with our proposal that this region of ize with m5 and m7 (42, 43). These results and their interpre-
that this region negatively regulates the activity of m8, a sug-
neurogenesis. Thus, Giebel and Campos-Ortega (67) propose that this region of bristle development (67). That this variant of m8 behaves as a dominant-negative, rather than a loss-of-function (as one would have predicted), suggests that the mutant protein might sequester endogenous wild-type m8, and possibly m5 and m7 as well, thus leading to enhanced phosphorylation. In this regard, a mutation that replaces Ser340 with Asp, abolishes its interaction with m3. It (42) report that a mutation in the proneural protein, Sc, that replaces Ser340 with Asp, abolishes its interaction with m3. It

Drosophila CKII Phosphorylates m5, m7, and m8

Fig. 7. Interaction of DmCKIIα with m8, m8S159A, and m8S159D. S. cerevisiae strain EGY048 harboring the LacZ-expression plasmid, pSH18-34, was transformed with plasmids expressing the indicated LexA fusion proteins in combination with either the activation domain alone (hatched bars) or the AD-DmCKIIα fusion protein (shaded bars). Transformants were grown in rich galactose media, and the levels of LacZ expressed were determined as described (48). β-Galactosidase (LacZ) activity is expressed in Miller units, and the data shown are the average of three independent experiments.

Theoretical and functional properties common to m5/7/8, and by extension those in D. hydei and by extension those in

Advances and progress in research on these proteins are more functionally related (42, 43, 59, 68) and that they are taken together with our results, it appears that neurogenesis as well as proneural proteins may be regulated by phosphorylation.

In summary, the data presented herein demonstrate that select members of the E(spl)/C, i.e. m5, m7, and m8, physically interact with DmCKII and are phosphorylated by this enzyme at an invariant Ser residue that is contained within a motif unique to these three isoforms. The suggestion that these three proteins are more functionally related (42, 43, 59, 68) and that the C-terminal domain of m8 acts to negatively regulate function in vivo (67) implicates the PEST motif and its resident CKII phosphorylation site. We believe that the data presented strengthen our contention for the presence of a new functional motif in these transcriptional repressors and raise the possibility that CKII may regulate neurogenesis via posttranslational modification of these proteins.

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Regina L. Trott, Madhavi Kalive, Zeev Paroush and Ashok P. Bidwai

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