Conserved role for the Dachshund protein with *Drosophila* Pax6 homolog Eyeless in insulin expression

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Members of the insulin family peptides have conserved roles in the regulation of growth and metabolism in a wide variety of metazoans. The *Drosophila* genome encodes seven insulin-like peptide genes, *dilp1–7*, and the most prominent *dilps* (*dilp2*, *dilp3*, and *dilp5*) are expressed in brain neurosecretory cells known as “insulin-producing cells” (IPCs). Although these *dilps* are expressed in the same cells, the expression of each *dilp* is regulated independently. However, the molecular mechanisms that regulate the expression of individual *dilps* in the IPCs remain largely unknown. Here, we show that Dachshund (Dac), which is a highly conserved nuclear protein, is a critical transcription factor that specifically regulates *dilp5* expression. Dac was strongly expressed in IPCs throughout development. *dac* loss-of-function analyses revealed a severely reduced *dilp5* expression level in young larvae. Dac interacted physically with the *Drosophila* Pax6 homolog Eyeless (Ey), and these proteins synergistically promoted *dilp5* expression. In addition, the mammalian homolog of Dac, Dach1/2, facilitated the promoting action of Pax6 on the expression of islet hormone genes in cultured mammalian cells. These observations indicate the conserved role of Dac/Dach in controlling insulin expression in conjunction with Ey/Pax6.

Members of the insulin-like peptides have evolutionarily conserved roles in the regulation of growth, energy homeostasis, stress resistance, life span, and fecundity (1–4). In *Drosophila*, seven insulin-like peptides (*Dilp1–7*) have been identified (5). All *Dilps* seem to be functionally equivalent agonists of the *Drosophila* insulin-like receptor (6). The major source of *Dilps* in *Drosophila* is a cluster of large neurons in a pair of medial neurosecretory cells, insulin-producing cells (IPCs). These cells primarily express *dilp2*, *dilp3*, and *dilp5* (6, 7). The expression and secretion of each *Dilp* into the hemolymph are strictly regulated throughout development (4–6, 8). The three *dilps* in the IPCs seem to be regulated independently at the transcriptional level. The expression of *dilp2* begins at the end of the embryonic stage, whereas *dilp5* and *dilp3* are expressed starting at the second and third instar, respectively (6). In addition to transcriptional control, the secretion of *Dilp* from IPCs is tightly regulated in response to nutrition levels (8). Moreover, there is signaling cross-talk among several *dilps* by positive or negative feedback regulation (9). All these features indicate the critical roles of *dilps* in animal development and energy homeostasis. Despite the important role of *Drosophila* IPCs in growth control, the molecular mechanism that controls the expression of individual *dilp* genes remains largely unknown.

The IPCs in the brain are derived from a single pair of neural stem cells at the embryonic stages (10, 11). Although the endocrine pancreas in mammals originates from the gut endoderm, remarkable analogies have been described between the *Drosophila* neurosecretory cells and the mammalian pancreatic islet cells (7, 12). Indeed, the differentiation of pancreatic β -cells involves the expression of many genes that also are expressed during neuronal development (13, 14). *Drosophila* Eyeless (Ey) has been reported to control IPC function, and this role is similar to that of the vertebrate homolog Pax6 in the pancreatic β -cells (15–17). Ey plays dual roles in IPCs; it controls neuronal differentiation and directly controls *dilp5* expression (17). However, it is unclear how a single factor

regulates two different processes in the IPCs during development. A previous study (10) demonstrated that a series of genes, *dachshund* (*dac*), *ey*, *optix*, and *tiptop* (*tio*), are expressed in the IPC lineage at the embryonic stage. Interestingly, all these genes function in a genetic regulatory module to initiate the development of the eye from epithelial tissue (18–20). In mammals, the genes that determine islet cell differentiation during embryogenesis control the tissue-specific expression of islet cell hormones in the adult. Therefore we tested whether genes that are involved in eye development regulate *dilp* expression at later stages of development.

Here, we report that Dac is a critical transcription factor that specifically regulates *dilp5* expression in young larvae. Dac interacts genetically and physically with Ey and synergistically promotes *dilp5* expression, suggesting that Dac specifies target gene expression via its interaction with Ey.

Results

Identification of *dac* as a Regulator of *dilp5* Expression. A previous study suggested that eye differentiation genes might be involved in the function of IPCs (10). To test this hypothesis, we screened the contribution of genes that are involved in eye development to the function of IPCs by using tissue-specific RNAi. We coexpressed *dilp2-Gal4*, which is an IPC-specific Gal4 driver (7), and Dicer-2 (*Dcr-2*) to enhance the knockdown efficiency (21). The expression levels of *dilp2*, *dilp3*, and *dilp5* were measured by real-time quantitative RT-PCR (qRT-PCR) using mRNA that was extracted from adult heads. Consistent with previous work (17), the knockdown of *ey* significantly suppressed *dilp5* expression (Fig. S1 and *SI Results and Discussion*). In addition to *ey*, we found that the knockdown of *dac* specifically suppressed *dilp5* expression (Fig. S1). The knockdown of *optix*, *tio*, *eye absent* (*eya*), *eye gone* (*eyg*), *twin of eyeless* (*toy*), or *sine oculis* (*so*) affected the expression of *dilps* either marginally or not at all. However, as expected, these RNAi lines induced obvious defects in eye morphology or decreases in mRNA levels (Fig. S1).

***dac* Specifically Regulates *dilp5* Expression.** To investigate a possible role of *dac* in IPCs, we first examined whether Dac is expressed in IPCs. Dac indeed was expressed in the IPCs throughout development (Fig. 1A). Because Dac already is expressed in the IPC lineage at the embryonic stage (10, 11), we examined whether *dac* is required for the initial specification and neuronal differentiation of IPCs. *dac*³ genetic-null homozygous mutants are lethal during the pupal stage, and these mutants exhibit severe defects in eye development (22, 23). However, the cell number, neuronal morphology, and projection patterns in the *dac*-mutant larvae were indistinguishable from those in the control (Fig. S2A and B).

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Fig. 1. *dac* is required for *dilp5* expression in IPCs during the early larval period. (A) Dac is expressed in brain IPCs during the larval and adult stages. The *dilp2-Gal4 > UAS-CD8-GFP* flies were immunostained for GFP and Dac. (Left) Reconstructed cross-sections of the z-series. (Right) Single confocal images. (Scale bars, 50 μ m.) (B) Developmental changes in the *dilp5* expression level in *dac* mutants. E, early; L, late; M, middle. (C) The percent difference in *dilp5* expression levels between the control and *dac* mutants, calculated from B. (D) Expression of Dilp5 in *dac* mutant IPCs. Control or *dac*-null mutant larvae were stained for GFP (green), Dilp5 (red), and nuclei (blue). (Scale bars, 10 μ m.)

In addition, *dilp2* expression was not impaired throughout larval development in the *dac* mutants (Fig. S2C). Because *dilp2* is first expressed at the late embryonic stage (7, 10), this result indicates that the IPCs are specified correctly at the embryonic stage and function normally in *dac*-null mutants.

We next monitored *dilp5* expression in the *dac* mutants during development. In the control larvae, *dilp5* was first expressed during the first instar, and *dilp5* expression peaked at the middle of the second instar (Fig. 1B). However, the *dac*³ homozygous mutants showed a strong reduction in *dilp5* expression during the first- and second-instar stages (Fig. 1B and C). The expression of *dilp5* in the *dac*³ mutants increased gradually, beginning at the end of the second instar, and reached normal levels at the middle of the third instar. We observed similar results in a transheterozygote of the *dac*³ allele with a deficiency covering the *dac* locus. Furthermore, a *dac*³ heterozygote and a transheterozygote of *dac*³ with a hypomorphic *dac*⁷ allele showed expression patterns that were intermediate between those of the control flies and the *dac*³ homozygote, suggesting that *dac* has a dose-dependent effect on *dilp5* expression. Notably, the IPCs of the central nervous system are the dominant source of *dilp5* signals at the developmental stages that we examined (Fig. S3). The reduction and recovery of the Dilp5 levels in the IPCs were confirmed further by immunostaining (Fig. 1D). Therefore, it is unlikely that the recovery of global *dilp5* expression at later larval stages in the *dac* mutants is the result of ectopic expression in other tissues.

Cell-Autonomous Requirement of *dac* and *ey* for *dilp5* Expression in IPCs. To confirm further the cell-autonomous function of *dac*, we analyzed *dilp5* levels in flies that expressed *dac*-RNAi in the IPCs. The knockdown of *dac* in the IPCs was verified by

immunostaining against Dac (Fig. S4A). Similar to the *dac* zygotic mutants, the reduction of *dac* in the IPCs led to a decrease of *dilp5* expression only in the young larvae but not in the third instar (Fig. 2A). These results indicate that *dac* is cell-autonomously required for *dilp5* expression in young larvae.

Ey binds directly to the *dilp5* promoter and controls *dilp5* expression (17). Therefore, we extended our time-course analysis to *ey* mutants. Both homozygous hypomorphic mutants of *ey*² and *ey*^R reduced *dilp5* expression in the second instar (Fig. 2B). However, similar to the *dac* mutants, *dilp5* expression was recovered in the third instar. These results were confirmed by the IPC-specific knockdown of *ey* (Fig. 2C and Fig. S4B). Importantly, *dilp2* expression was not changed in the absence of Dcr-2, indicating that the partial reduction of *ey* affects *dilp5* expression but does not abrogate the differentiation or function of IPCs. In contrast, a strong RNAi line against *ey* (RNAi #3) caused a severe reduction in *dilp5* and *dilp2* expression in all larval stages, even in the absence of Dcr-2 (Fig. 2C), indicating that *ey* plays a critical role in the differentiation and/or normal function of IPCs (SI Results and Discussion) (17).

We next asked whether the overexpression of *dac* promotes *dilp5* expression. However, this gain-of-function approach was not informative. The overexpression of *dac* in IPCs significantly reduced *dilp5* expression (Fig. S5A). Under these conditions, the expression of *dilp2* and *dilp3* also was decreased, and the flies showed a significantly smaller body size (Fig. S5B). Therefore, it is likely that ectopic *dac* does not promote *dilp5* expression but rather impairs *dilp5* expression and the normal function of IPCs. Similarly, the overexpression of *ey* in IPCs has been demonstrated to reduce body weight (17). Because both *dac* and *ey* are haploinsufficient with respect to *dilp5* expression (Fig. 1B) (17),

Fig. 3. Dac and Ey synergistically promote *dilp5* expression. (A and B) The expression of Ey (A) or Dac (B) was analyzed in IPCs of the indicated genotypes. The IPCs were identified by Dilp2 staining. Single confocal images are shown. (Scale bars, 10 μ m.) (C) *dac* interacts genetically with *ey*. *dilp5* transcripts were analyzed by qRT-PCR in flies of the indicated genotypes. (D) *dilp5* expression was analyzed by qRT-PCR in S2 cells using a *dilp5* genomic reporter. The coexpression of Dac and Ey synergistically promotes *dilp5* transcription. (E) Luciferase activity was analyzed in S2 cells using the *dilp5* promoter that controlled the expression in IPCs (6). (F) The interaction of Ey and Dac with the *dilp5* promoter was analyzed by ChIP-qPCR. (Upper) GFP-tagged proteins were precipitated in the absence or presence of Flag-tagged Ey/Dac. (Lower) Input samples. All values are means and SD ($n = 3-4$). * $P < 0.05$; ** $P < 0.01$ (Student's *t* test).

2 toward islet hormone gene expression remains unknown. To determine whether the synergistic action of Dac and Ey is conserved in mammals, we analyzed reporter gene expression using

the *Insulin* and *Glucagon* promoters. The expression of Pax6 slightly promoted the rat *Insulin-1* reporter in HEK293T cells (Fig. 5A). Pax6 significantly up-regulated the rat *Glucagon*

Fig. 4. Dac interacts physically with Ey and with itself through different regions. (A) Coimmunoprecipitation of full-length Dac and Ey from transfected HEK293T cells. Flag-tagged proteins were analyzed in the immunoprecipitate (IP) of the GFP proteins. WB, Western blot. (B) (Upper) Dac interacts with the PD of Ey. (Lower) The domain structure and deletion constructs of Ey are shown. Numbers refer to amino acids. (C) The DD1 domain of Dac interacts with Ey, whereas the DD2 domain of Dac facilitates self-association. (D) EMSA showing the interaction of Ey and Dac with the biotin-labeled *dilp5* promoter in vitro.

reporter, as reported previously (15). Conversely, the expression of Dach1 or Dach2 had only marginal effects on the *Insulin-1* and *Glucagon* reporters. However, the coexpression of Pax6 with either Dach1 or Dach2 further promoted expression from the *Insulin-1* and *Glucagon* promoters (Fig. 5A). As in *Drosophila*, Pax6 interacted with Dach1 (Fig. 5B). Dach1 also was coimmunoprecipitated with Dach1 and Dach2. Taken together, these results indicate that mammalian Dach1 or Dach2 enhances the transcriptional activity of Pax6 on islet hormone genes, presumably through the formation of a protein complex.

To examine further whether Dach1/2 plays a role in *Insulin* expression, we used a β -cell-derived rat insulinoma cell line, Rin-m, which expresses high levels of *Insulin-1*. We detected the expression of *Dach1*, *Dach2*, and *Pax6* in these cells by qRT-PCR. The expressions of these genes were largely suppressed by introducing siRNA against *Pax6*, *Dach1*, or *Dach2* (Fig. 5C).

Fig. 5. Mammalian Dach1/2 binds to Pax6 and controls *Insulin* expression. (A) Luciferase activity was analyzed in HEK293T cells by using a rat *Insulin-1* or rat *Glucagon* promoter. (B) Coimmunoprecipitation of Dach1 with Pax6 from HEK293T cells expressing the indicated constructs. Flag-GAPDH was cotransfected as a negative control for the immunoprecipitation assays. (C) Knockdown of Pax6 and Dach1/2 in Rin-m cells. The expression levels of *Pax6*, *Dach1*, and *Dach2* were suppressed by introducing siRNA, as assessed by qRT-PCR. *Insulin-1* expression levels were analyzed in Rin-m cells that had been transfected with the indicated siRNA. (D) The interactions of Pax6 and Dach1 with the *Insulin-1* promoter were analyzed in Rin-m cells by ChIP-qPCR. All values are means and SD ($n = 3$). * $P < 0.05$; ** $P < 0.01$ (Student's t test).

These knockdown cells expressed reduced levels of *Insulin-1*. In addition, both Pax6 and Dach1 bound to the endogenous *Insulin-1* promoter (Fig. 5D), indicating that Dach1/2 and Pax6 are required for the normal expression of *Insulin-1* in Rin-m cells.

Although *Drosophila* contains a functional Glucagon analog, adipokinetic hormone, which is produced by corpora cardiaca cells in the ring gland (12), neither Ey nor Dac was expressed in the ring gland during development (Fig. S9). Nevertheless, our results indicate an evolutionarily conserved role for Dac/Dach in enhancing the effect of Ey/Pax6 on endocrine hormone gene expression.

Discussion

Regulation of *dilp5* Expression During Development. In the present study, we demonstrated the essential role of *dac* for *dilp5* expression in young larvae. The function of *dac* in IPCs likely is critical for the regulation of growth and energy homeostasis, although these roles normally are masked by the presence of other *dilps* and feedback systems (Fig. S10 and *SI Results and Discussion*). Our results also revealed the presence of additional mechanisms that regulate *dilp5* expression in the last instar. However, it is unclear why additional regulation is required during the last instar. During larval development, *Drosophila* undergoes two molting stages to facilitate body growth. The commitment of various tissues to pupal development occurs early during the last larval instar of many holometabolous insects, including *Drosophila* (2, 26). In addition, after a species-specific size known as the “critical weight” has been achieved during the last instar, a nutrition-independent mode of development is initiated in the imaginal discs to promote puparium formation (27). Therefore, the primary function of *dac* and *ey* may be to control *dilp5* expression in a nutrient-responsive fashion during early larval stages. Further dissection of the molecular control of *dilp5* expression in the last instar will provide a framework for understanding the switch from nutrition-dependent to nutrition-independent modes of growth for pupal commitment.

Synergistic Action of Dac and Ey for Gene Transactivation. Our expression analyses indicated that the *dac* is haploinsufficient with respect to *dilp5* levels. However, the overexpression of *dac* in IPCs caused a severe reduction of *dilp5* expression. Similarly, the loss of one copy of *ey* partially reduces the level of *dilp5*, whereas the ectopic expression of *ey* impairs body growth (17). In mammals, Pax6 is a key regulator of organogenesis of the eye, pancreas, and brain (28, 29). Functional studies have demonstrated that the level of Pax6 protein is important for determining the developmental outcome. Both Pax6 loss of function and gain of function in mice cause ocular abnormalities and defects in the pancreatic islets (16, 28–30). Thus, the functions of Ey and Dac in IPCs are critically dependent on a defined protein threshold.

Biochemical analyses indicated that Dac interacted physically with itself and with Ey. A structure-function analysis in *Drosophila* revealed that the DNA-binding DD1 domain is necessary for the function of Dac, whereas DD2 is not always required but assists the function of DD1 (22). It has been suggested that dimer formation through DD2 increases the efficiency of DD1 or DNA-bound transcription factors and thus amplifies transactivator functions. Considering that Dac cannot stimulate *dilp5* expression on its own, Dac is likely to be recruited to the promoter via association with Ey in vivo. The greater recovery of *dilp5* promoter upon the coexpression of Ey and Dac supports this notion. Mammalian *Insulin* expression is regulated by several transcription factors, such as Pdx-1, MafA, and NeuroD1 (13, 14, 31). These proteins occupy distinct regulatory elements within the *Insulin* promoter and promote transcription in a coordinated and synergistic manner. Because the levels of *dac* and *ey* are critical for *dilp5* expression, Dac dimer formation most likely enables the formation of a higher-order regulatory complex that amplifies activation signals.

In mammals, the production and secretion of insulin and glucagon from the pancreas are crucial for glucose homeostasis. Mutations in the *Pax6* locus cause impaired insulin secretion, early-onset diabetes mellitus, and aniridia (16, 32, 33). A recent genome-wide *in silico* promoter analysis identified *Glucagon* and *Igf2* as potential targets of mammalian *Dach1* (34). In addition, a diet-induced β -cell-dysfunction analysis in mice identified that the altered expression of several genes, including *Dach1*, is correlated significantly with data from genome-wide association studies for type 2 diabetes in humans (35). Given the conserved roles of *Ey/Pax6* and *Dac/Dach* on *Dilp*/insulin production in *Drosophila* and mammals, further investigation of the function of mammalian *Dach* in the pancreatic islet likely will provide insight into the molecular control of glucose homeostasis.

Materials and Methods

Materials and methods are described in detail in *SI Materials and Methods*.

***Drosophila* Strains and Plasmids.** The following stocks were used: *w¹¹¹⁸* (used as a control), *dac³*, *dac⁷*, *UAS-dac* (from G. Mardon, Houston, TX), *dilp2-Gal4* (from E. Rulifson, San Francisco, CA), *Df(2)Exel7066* (a deficiency for the *dac* locus), *OK107-Gal4*, *UAS-mCD8-GFP*, *UAS-Dcr-2*, *ey²*, *ey^R* (from Bloomington Stock Center). Details of the RNAi lines used in this study are given in *SI Materials and Methods*.

Immunohistochemistry. Adult or larval brains were dissected in PBS and fixed for 10 min in 3.7% (vol/vol) formaldehyde in PBS containing 0.2% TritonX-100 and processed as previously described (36). The following primary antibodies

were used: mouse anti-Dac, mouse anti-Ey (Developmental Studies Hybridoma Bank), and chick anti-GFP (Abcam). The secondary antibodies were Alexa 488- and Alexa 555-conjugated antibodies (Invitrogen). The nuclei were stained with Hoechst 33342. The *Dilp2* and *Dilp5* antibodies were generated in rabbits against the peptides CEEYNPVIPHK and CPNGFNSMFAK, respectively. The images were acquired with a Zeiss LSM700 confocal microscope.

Binding Assays. For immunoprecipitations and pull-downs, the HEK293T cells transfected with intended plasmids were processed as described (36). Antibodies used in immunoprecipitation and Western blotting were rabbit anti-GFP (MBL International), mouse anti-Flag (Sigma), and mouse anti-GFP (Roche).

qRT-PCR Analysis. qRT-PCR was performed essentially as described previously (37). Total RNA was prepared using TRIzol (Invitrogen) or the RNeasy mini kit (QIAGEN) and RNase-Free DNase Set (QIAGEN), and reverse transcription was performed using the PrimeScript RT reagent kit (Takara Bio). qRT-PCR was performed on an ABI PRISM 7500 real-time PCR system (Applied Biosystems) using SYBR Premix Ex TaqII (Takara Bio).

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