Phosphorylation by the DHIPK2 Protein Kinase Modulates the Corepressor Activity of Groucho*

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Groucho function is essential for Drosophila development, acting as a corepressor for specific transcription factors that are downstream targets of various signaling pathways. Here we provide evidence that Groucho is phosphorylated by the DHIPK2 protein kinase. Phosphorylation modulates Groucho corepressor activity by attenuating its protein-protein interaction with a DNA-bound transcription factor. During eye development, DHIPK2 modifies Groucho activity, and eye phenotype generated by overexpression of Groucho differs depending on its phosphorylation state. Moreover, analysis of nuclear extracts fractionated by column chromatography further shows that phospho-Groucho associates poorly with the corepressor complex, whereas the unphosphorylated form binds tightly. We propose that Groucho phosphorylation by DHIPK2 and its subsequent dissociation from the corepressor complex play a key role in relieving the transcriptional repression of target genes regulated by Groucho, thereby controlling cell fate determination during development.

Transcriptional repression is an essential mechanism in the control of gene expression (1, 2). In general, DNA-bound transcriptional repressors recruit corepressors to maintain the inactive state of target genes. The Drosophila Groucho protein is the founding member of a family of corepressors that includes the human transducin-like enhancer of split (TLE) proteins (3, 4). Groucho itself does not bind to DNA but is recruited by DNA-bound repressors via an inherent protein motif, thereby serving as a corepressor for such transcription factors as Hairy (5), Runt (6), Engrailed (5, 7), Dorsal (8), dTCF (9), Huckebein (10), Tinman/NK-4 (11), and Bap/NK-3 (12), all of which play important roles throughout development.

Groucho participates in a wide array of developmental processes including segmentation, neurogenesis, sex determination (10, 13), and patterning of the nonsegmental termini of the Drosophila embryo (14). Indeed, the interaction of Groucho with basic helix-loop-helix proteins of the E(spl) family is believed to mediate at least some of the functions of the Notch signaling pathway (13). The loss-of-function mutation of groucho suppresses a wingless and armadillo mutant phenotype, and reduced levels of maternal Groucho severely impair the ability of dTCP to repress transcription (9), implicating Groucho in the Wingless/Wnt signaling pathway (15). Groucho also participates in terminal development by restricting the expression of tll and hkb to the embryonic termini (10, 14), and Torso receptor-tyrosine kinase signaling permits terminal gap gene expression by antagonizing Groucho-mediated repression (14, 16). Recently, it has been shown that Groucho acts as a corepressor of the transcriptional repressor Brinker, which antagonizes Dpp-mediated gene activation (17, 18). These findings clearly illustrate the important role for Groucho in these signaling pathways. However, molecular mechanisms of how Groucho functions and how Groucho activity is regulated remain unclear.

One obvious function of Groucho in these diverse developmental pathways is to act as a global long range corepressor to maintain the repression state of target gene expression (2, 19, 20). To this end, Groucho may function by recruiting histone deacetylases to produce a large transcriptionally silent chromosomal domain (12, 20, 21). Upon the activation of these signaling pathways, however, Groucho-mediated transcriptional repression has to be relieved (2, 22). In addition, most transcription factors that recruit Groucho as a corepressor can also act as transcriptional activators (6, 9, 11, 23, 24), suggesting that, depending on either target gene or developmental context, these transcription factors are able to interact with both coactivators and corepressors. In any case, it is conceivable that there may be critical on-off regulatory switches that involve Groucho and other coregulators (2).

Homeodomain-interacting protein kinase 2 (HIPK2) is a member of the protein kinase family that acts as a coregulator for various transcription factors (25, 26). We have shown that HIPK2 is a component of the corepressor complex recruited by the NK-3 homeodomain transcription factor, which also includes Groucho and histone deacetylase HDAC1 (12). Importantly, HIPK2 physically interacts with Groucho and appears to regulate the corepressor activity of the protein in cultured cells. Because Groucho is a phosphoprotein (27) and is also known to interact with histone deacetylase (12, 21), we hypothesized that its phosphorylation status, as determined by HIPK2, acts as a potential on-off switch to relieve transcriptional repression mediated by Groucho.

In this study, we demonstrate that Groucho is an in vivo target for HIPK2, a Drosophila homologue of the mammalian HIPK2. We further investigated the functional role of this

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The abbreviations used are: TLE, transducin-like enhancer of split; HIPK2, homeodomain-interacting protein kinase 2; aa, amino acid(s); GST, glutathione S-transferase; GFP, green fluorescent protein.
protein modification in the regulation of the corepressor activity of Groucho both in vivo and in vitro. Our results show that the phosphorylation of Groucho modulates its corepressor activity by attenuating protein-protein interaction with a DNA-bound transcription factor. Analysis of nuclear extracts fractionated by column chromatography further shows that unphosphorylated Groucho associates tightly with a corepressor complex, whereas phospho-Groucho dissociates from the corepressor complex. Our results provide evidence that Groucho phosphorylation by DHIPK2 and its subsequent dissociation from the corepressor complex play key roles in relieving the transcriptional repression of target genes regulated by Groucho during development.

MATERIALS AND METHODS

Expression Vectors and Reporter Plasmids—Full-length Drosophila hikp2 (hikp2) cDNA (CG17090) (28) was obtained from expressed sequence tag clones and verified by nucleotide sequencing.2 Cloning sites (EcoRI site before the start codon and XbaI site after the termination codon) were introduced by PCR with specific primers, and the DNA fragments were cloned into the corresponding sites of the pEGFP-C2 expression vector to construct the DHIPK2 plasmid. A mutation (lysine to arginine at position 467) (29) was generated by PCR-based mutagenesis with specific primers to construct the kinase-inactive DHIPK2 expression vector, DHIPK2(KR). The DHIPK2(KD) plasmid encodes alanines 1–629, including the kinase domain. The DHIPK2(KD) was constitutively active in catalytic activities as mouse HIPK2(KD) (25, 26). Full-length eyeless cDNA was obtained by combining the EcoRI-Stul DNA fragment (336-bp DNA fragment containing exons 1 and 2) amplified from a Drosophila embryo cDNA library (Clontech) with the Stul-Xhol DNA fragment (2.6 kb containing exons 3–9) from expressed sequence tag clone (GH101157). The resulting full-length eyeless cDNA construct includes all exons encoding 898 amino acid residues (29) and was inserted into the EcoRI/SalI sites of the pCS3+ plasmid (13) (for Myc tagging and in vitro translation) or the pGEX-5X-1 plasmid, and fusion proteins were expressed and purified as described previously (33).

Generation of Transgenic Fly Lines—DNA fragments encoding the wild type Groucho and mutant Groucho, GRO(SA), and GRO(SE) were excised from pCI-GRO, pCI-GRO(SA), and pCI-GRO(SE), respectively, and introduced into the EcoRI/Xhol sites of the P-element vector pUAST (34). For the constructions of P-elements containing the wild type DHIPK2, the constitutively active HIPK2(KD), and the kinase-dead HIPK2(KR), the NehI/Xhol DNA fragments from the corresponding expression vectors were excised from the P-elements and ligated into the XbaI site of the pUAST. Transgenic lines harboring the UAS-DNA constructs were established using standard procedures as described (35). Five different transgenic lines each for wild type GRO and GRO(SA) and GRO(SE) mutants were established, and at least two different transgenic lines were crossed with the ey-GAL4 driver line to see the potential variations of phenotypes by each transgenic line. Fly growth and crosses of transgenic lines were performed at 25 °C by standard procedures. ey-GAL4 (36) driver lines were obtained from the Bloomington Stock Center.

Antibodies and Immunohistochemistry—Anti-GRO and anti-DHIPK2 rabbit polyclonal antibodies were raised with gel-purified GST-GRO (aa 1–333) and GST-DHIPK2 (aa 561–932) (as described (37). Phosphopeptide, CKSSRSRPSLSLTKR (aa 291–302), was synthesized and used for immunization of rabbits after conjugation to KLH. Anti-phosphopeptide-specific Groucho antibodies were purified using a phosphopeptide affinity column after preabsorption of serum on a nonphosphopeptide column (BIOSOURCE International). Eye-antennal imaginal discs were dissected and stained as described previously (38). The following antibodies were used: anti-Elav (39), anti-Eya (40), and anti-Dac monoclonal antibodies (41).

RESULTS

Phosphorylation of Groucho by DHIPK2—We have previously shown that Groucho and HIPK2 are components of the corepressor complex recruited by NK-3 (12). Interestingly, we have observed that a kinase-inactive mutant of HIPK2, but not wild type HIPK2, can enhance Groucho corepressor activity (12). These results indicate that the kinase activity is involved in this regulation and suggest that HIPK2 may regulate the corepressor activity of Groucho by direct phosphorylation. To

2 Y-O. Kim, C. Y. Choi, and Y. Kim, unpublished results.
Regulation of Groucho Activity by DHIPK2

Fig. 1. Groucho is phosphorylated by DHIPK2. A, phosphorylation of Groucho by DHIPK2 in cultured cells. Cells were co-transfected with groucho and dhipk2 expression vectors, and cell extracts treated with (lane 4) or without calf intestinal alkaline phosphatase were analyzed by Western blot with anti-Myc antibody. p-GRO, phospho-Groucho; GRO, unphosphorylated Groucho. B, in vitro phosphorylated Groucho by DHIPK2 was subjected to phosphoamino acid analysis. Phosphoserine (p-S), phosphothreonine (p-T), and phosphotyrosine (p-Y) amino acids were determined with phosphoamino acid standards. C, in vitro mapping of potential phosphorylation sites by DHIPK2. Various GST-Groucho fusion proteins were phosphorylated with DHIPK2 in vitro and analyzed with autoradiography after SDS-gel electrophoresis. Constructs used for analysis are shown under the schematic diagram of Groucho. The arrowhead indicates the DHIPK2 band caused by autophosphorylation, and various Groucho proteins are marked with a bracket (GRO). D, conservation of a phosphorylation site in Groucho. One of the phosphorylation sites is compared with amino acid sequences from corresponding regions of human TLE proteins (human TLE1, BC015747; human TLE3, NM_005078). The site of phosphorylation for generation of a phosphopeptide antibody is marked with an asterisk. E and F, phosphopeptide analysis of wild type and mutant Groucho proteins in vitro (E) and in vivo (F). E, mutations (Ser to Ala) of phosphorylation sites (194/196, 285/287, and 297) were generated, and Groucho proteins were subjected to phosphopeptide analysis after phosphorylation by DHIPK2 in vitro. F, either wild type or mutant groucho expression vector was cotransfected with a dhipk2 expression vector, and cells were labeled with 32P. Immunoprecipitated proteins with an anti-Groucho antibody were resolved by SDS-polyacrylamide gel electrophoresis, and the eluted band was subjected to phosphopeptide analysis. G, Groucho is phosphorylated in vivo. Drosophila embryo extracts were treated with phosphatase and subjected to Western blot analysis with an anti-Groucho antibody (lanes 1–3) and anti-phosphopeptide antibody (lanes 4–6).

test this hypothesis, we cotransfected cells with Myc-Groucho and DHIPK2 expression vectors, and Groucho proteins were analyzed by Western blot with an anti-Myc antibody. As shown in Fig. 1, a slowly migrating band was detected in extracts of cells cotransfected with wild type DHIPK2 (Fig. 1A, lane 3). This band (p-GRO) was not detected in extracts of cells either cotransfected with the kinase inactive mutant DHIPK2(KR) (lane 2) or treated with a phosphatase (lane 4), suggesting this slowly migrating band is a phosphorylated form of the Groucho protein. Phosphoamino acid analysis revealed that practically all of the phosphorylation was confined to serine residues (Fig. 1B).

Using Groucho proteins and DHIPK2 expressed in E. coli we mapped potential phosphorylation sites of the protein in vitro (Fig. 1, C–E). Initially, we found that the middle portion of Groucho is strongly phosphorylated by DHIPK2 (Fig. 1C, lane 1). In contrast, the WD40 repeat region or the amino-terminal region of the protein was not phosphorylated (Fig. 1C, lanes 6 and 7). Further serial deletions lacking the serine residue at the aa 297 site greatly reduced in vitro phosphorylation of Groucho (Fig. 1C, lanes 4, 5, and 8–11), suggesting that the aa 297 site is one of major sites phosphorylated by DHIPK2.

Two-dimensional phosphopeptide analyses of each deletion mutants defined three potential major phosphorylation sites (serine residues at positions 194/196, 285/287, and 297) (see below). These phosphorylation sites reside within the CcN domain, which contains potential CDC2 kinase and casein kinase II phosphorylation sites (42) and the serine and proline-rich (SP) domain of Groucho (Fig. 1, C and D). These phosphorylation sites (Ser-196, Ser-287, and Ser-297) are well conserved among the mammalian TLE family proteins. Among them, the amino acids near Ser-297 were compared with the corresponding regions of human TLE proteins (Fig. 1D). To further confirm the phosphorylation sites, we generated mutant Groucho in which potential phosphorylation sites were replaced by alanine residues (gro(SA)). After phosphorylation with DHIPK2, Groucho proteins were subjected to two-dimensional phosphopeptide analysis (Fig. 1E). At least seven spots were detected in wild type Groucho (Fig. 1E, left). Among them, four spots (spots 1, 3, 4, and 7) disappeared in mutant Groucho (Fig. 1E, middle panel). In cultured cells, the same spots were not detected (Fig. 1F, right panel), suggesting that these sites are indeed phosphorylated by DHIPK2. We detected phospho-Groucho in the embryo extracts (Fig. 1G, lane 4) using an
antibody generated by a phosphopeptide containing phospho-
serine at the conserved Ser-297 phosphorylation sites (Fig. 1D).
This band disappeared after phosphatase treatment (lanes 5 and 6). Taken together, these results indicate that Groucho is phosphorylated by DHIPK2 both *in vitro* and *in vivo*.

**Effect of Groucho Phosphorylation during Eye Development**—To test whether phosphorylation of Groucho is functionally relevant *in vivo*, we generated transgenic flies harboring either the wild type or mutant Groucho. In addition to the mutant Groucho containing serine to alanine substitutions (gro(SA)), we also generated transgenic flies harboring the mutant Groucho containing serine to glutamic acid substitutions (gro(SE)), which may mimic the phosphorylation status of Groucho. Because the *Drosophila* eye system is very useful for analysis of phenotypes and because phosphorylated Groucho was detected in the eye-antennal disc (data not shown), we ectopically expressed either the wild type or mutant Groucho in the eye-antennal disc using the GAL4-UAS binary system (34) and analyzed the resulting eye phenotypes. Unexpectedly, transgenic flies harboring ey-GAL4 and UAS-groucho showed the eyeless phenotype (Fig. 2B). Mutant Groucho (gro(SA)), which is an unphosphorylated form of Groucho, also showed the eyeless phenotype (Fig. 2C). However, transgenic flies harboring ey-GAL4 and UAS-groucho showed the eyeless phenotype (Fig. 2B). This band disappeared after phosphatase treatment (lanes 5 and 6). Taken together, these results indicate that Groucho is phosphorylated by DHIPK2 both *in vitro* and *in vivo*. Although both the wild type and the mutant Groucho (gro(SE)) showed similar eyeless phenotypes, the defect in the head capsule of transgenic flies harboring mutant Groucho (gro(SE)) was more severe (Fig. 2G) than that in the transgenic flies harboring wild type Groucho (Fig. 2F). The head morphology appeared normal in the ey-gro(SE)-expressing transgenic fly (Fig. 2H). In
order to rule out the possibility that the different phenotypes were caused by different expression levels of wild type and mutant Groucho, the expression levels of Groucho were checked by crossing each groucho-transgenic line with a paired-GAL4 driver line and Western blotting using anti-GRO antibodies. There were no great differences in expression levels between wild type and mutant Groucho (data not shown).

To confirm these eye phenotypes, eye-antennal discs were isolated from the third instar larvae and immunostained with an anti-Elav antibody, which detects photoreceptor cells in the eye disc. Consistent with the eye phenotypes, no staining was detected in the discs from both wild type and mutant groucho (data not shown). Occasionally, we observed an additional tiny eye in the midsized eye disc from the mutant groucho (Fig. 2, J and K). Occasionally, we observed an additional tiny eye in the midsized eye disc from the mutant groucho (Fig. 2, J and K). The size of the eye disc was also greatly reduced (Fig. 2, J and K, arrow). These results indicate that phosphorylation of Groucho is functionally relevant in vivo.

In order to investigate the genetic interaction between groucho and dhipk2 in vivo, we also generated transgenic flies harboring P-elements encoding various GFP-DHIPK2 fusion proteins. Overexpression of the constitutively active form of dhipk2(KD) in the eye disc using ey-GAL4 produced a midsized eye (Fig. 2N). Occasionally, we observed an additional tiny eye containing a small number of ommatidium (Fig. 2N, arrowhead). Furthermore, overexpression of both groucho and dhipk2(KD) suppressed the groucho phenotype (Fig. 2O). These results indicate that dhipk2 modifies groucho gene activity in vivo. Consistent with these results, overexpression of the kinase-inactive dhipk2(KR) produced either a very small eye phenotype (Fig. 2P) or occasionally the eyeless phenotype mimicking the groucho phenotype (data not shown). Overexpression of wild type DHIPK2 did not produce any visible change in the eye (data not shown), suggesting that activation of DHIPK2 may depend on developmental signaling pathways.

Interaction of Groucho with the Eyeless Transcription Factor—Eyeless, a member of the paired domain protein family, is a master regulator of eye development (43). The loss of Eyeless function causes the eyeless phenotype. On the other hand, forced expression of Eyeless in other imaginal discs produces an ectopic eye. The eyeless phenotype generated by the overexpression of Groucho in the eye-antennal disc prompted us to test whether Groucho can affect transcriptional activity of the Eyeless transcription factor (Fig. 3). Indeed, in the eye disc harboring ey-GAL4 and UAS-groucho, expression of the Eyeless target genes eyes absent (eye) and dachshund (dac) was severely disrupted (Fig. 3A). However, expression of these genes was not changed in the antennal disc. In transient expression assays using either the reporter (SOTK-Luc) containing the endogenous so enhancer, which includes the Eyeless target sequence (30), or the reporter (CD19TK-Luc) containing the synthetic Eyeless binding sites (31), Eyeless enhanced reporter gene expression (Fig. 3B, lanes 2 and 7). This effect is alleviated by coexpression of Groucho (Fig. 3B, lanes 3–5 and 8–10). These data suggest that Groucho inhibits the transcriptional activity of Eyeless both in vivo and in cultured cells.

Next, we tested whether Eyeless and Groucho can interact with each other. In cultured cells, Groucho interacts with Eyeless, which was demonstrated by coinmunoprecipitation (Fig. 3C). Furthermore, GST pull-down assays revealed direct physical interaction between the two in vitro (Fig. 3, D and E). Two different portions of Eyeless can interact with the amino-terminal portion of Groucho (Fig. 3, E and F). One is the amino-terminal domain of Eyeless, which includes the paired domain (Fig. 3D, lane 7); the other is the carboxyl-terminal region of the Eyeless protein (Fig. 3D, lane 10). The truncated form of Eyeless, in which these regions are deleted, failed to show interaction with Groucho (Fig. 3D, lane 14). The carboxyl-terminal domain contains the YSPW motif, which shows homology to the known consensus motif ((W/Y)(P)(W/Y)) for Grou-
Groucho binding. Indeed, deletion or mutation of this motif abolished interaction with Groucho (Fig. 3D, lanes 19 and 20). These results indicate that Groucho down-regulates the transcriptional activity of Eyeless by physical interaction with this eye master regulatory protein.

**Phosphorylation by DHIPK2 Modulates the Coactivator Activity of Groucho**—Activities of transcription factors are modulated by phosphorylation (44). However, less is known about corepressor molecules as to whether their transcriptional activities are regulated by phosphorylation. Because DHIPK2 can phosphorylate Groucho (Fig. 1) and because phosphorylation can affect its activity in vivo (Fig. 2), we further investigated the role of DHIPK2 phosphorylation in regulation of the corepressor activity of Groucho. Eyeless can act as either a transcriptional activator or repressor (45). In fact, Eyeless contains both a repressor domain (aa 1–230) and an activator domain (aa 531–898), as shown by testing with GAL4 fusion constructs (data not shown). Interestingly, these domains include Groucho interaction domains (Fig. 3). In the absence of Groucho, Eyeless showed a modest transcriptional activation of the reporter gene when fused to the GAL4 DNA binding domain (Fig. 4A, lane 2). Consistent with our previous results (Fig. 3B), coexpression of Groucho inhibited reporter gene activation (Fig. 4A, lane 3). However, coexpression of DHIPK2 greatly enhanced reporter gene activation (Fig. 4A, lane 4). Also, coexpression of DHIPK2 could relieve the inhibitory effect of Groucho on Eyeless (Fig. 4A, lane 6). In contrast, coexpression of the kinase-inactive DHIPK2(KR) failed to enhance reporter gene activation. Instead, DHIPK2(KR) down-regulated reporter gene expression (Fig. 4A, lane 5) and could not overcome the inhibitory effect of Groucho (Fig. 4A, lane 7). These results suggest that DHIPK2, but not the kinase-inactive DHIPK2(KR), can enhance transcriptional activation of Eyeless and can relieve transcriptional repression mediated by Groucho, presumably by phosphorylation of either Eyeless or Groucho.

In cultured cells, both Eyeless and Groucho can be phosphorylated by DHIPK2, because slowly migrating bands are detected only in cell extracts cotransfected with DHIPK2 (Fig. 4, B, lane 2, and C, lanes 2, 4, and 6). Coimmunoprecipitation experiments revealed that, in the presence of DHIPK2, Eyeless could interact with Groucho less efficiently than in samples cotransfected with DHIPK2(KR) (Fig. 4B, middle panel, lanes 5 and 6), suggesting that phosphorylation of either Groucho or Eyeless could affect protein-protein interaction. In order to clarify the effect of phosphorylation on protein-protein interaction between Eyeless and Groucho, extracts were prepared from cells cotransfected with either Eyeless or Groucho in the presence or absence of DHIPK2 and subjected to GST pull-down assays with GST-GRO (lanes 3 and 4) or with GST-EY (lanes 7 and 8). WT, wild type.

**Phosphorylation of Groucho Promotes Its Dissociation from the Coactivator Complex**—The direct effect of phosphorylation of Groucho on its coactivator activity was further tested with the mutant Groucho, GRO(SA) and GRO(SE) (Fig. 5, A–C). We have used the GAL4-EYAD construct, in which the transactivation domain of Eyeless is fused to the GAL4 DNA binding domain (Fig. 5A, lane 2) in order to measure the effect of the coactivator activity of mutant Groucho proteins more efficiently. The activation domain of Eyeless (EYAD) also contains the Groucho interaction motif, YSPW (Fig. 3F). Coexpression of the mutant Groucho, GRO(SA), suppressed EYAD transactivation more efficiently (Fig. 5A, lane 4), whereas the mutant Groucho GRO(SE) was less efficient in suppressing the EYAD transactivation when compared with the wild type Groucho (Fig. 5A, lane 5). The suppression of EYAD-mediated transactivation by Groucho was exerted through the Groucho-interacting motif (YSPW) of EY, in which substitution of YSPW motif to ASAA abrogated Groucho-mediated suppression of transcription (Fig. 5A, lanes 10–17). The relief of transcriptional repression of GRO(SA) by
DHIPK2 was less efficient than that of the wild type Groucho or GRO(SE) (Fig. 5A, lanes 7–9). These results suggest that the mutant GRO(SE), which may mimic the phosphorylated form of Groucho, could not be efficiently recruited by the DNA-bound Eyeless transcription factor. In fact, in cotransfected cells, GRO(SE) is less efficiently recruited by Eyeless (Fig. 5B, lanes 1–3). In the absence of DHIPK2, both wild type Groucho and the mutant GRO(SA) are equally well recruited by Eyeless (Fig. 5B, lanes 1 and 2). Consistent with the results of transient expression assays (Fig. 5A), coexpression of DHIPK2 decreased precipitation of wild type Groucho (Fig. 5B, lane 4). In addition, in vitro GST pull-down assays showed that GRO(SE) interacts with Eyeless less efficiently (3-fold decrease) (Fig. 5C, top). Because Groucho can interact with histone deacetylase HDAC1 (12, 21), we also investigated whether phosphorylation of Groucho can affect protein–protein interaction of Groucho with other components of the corepressor complex using GAL4-Groucho constructs. GAL4-GRO showed corepressor activity, and this activity was relieved by expression of DHIPK2 (Fig. 5B, lanes 19 and 22), whereas corepressor activity of GALA-GRO(SE) was not diminished by
DHIPK2 expression (Fig. 5A, lanes 20 and 23). Also, GALA-GRO(SE) showed decreased corepressor activity in cultured cells (Fig. 5A, lane 21). In vitro, GRO(SE) interacts less efficiently with histone deacetylase HDAC1 (Fig. 5C, bottom). These results indicate that phosphorylation of Groucho by DHIPK2 decreases protein-protein interaction with both Eyeless and histone deacetylase HDAC1, consequently reducing recruitment of Groucho to participate in transcriptional repression.

Using a gel filtration column, we fractionated nuclear extracts from either Drosophila embryos or transfected cells, and fractions were analyzed by Western blot (Fig. 5D). Since Groucho was shown to form oligomers (46), Groucho was detected in fractions larger than its monomer size. However, Groucho was also detected in fractions with a molecular mass larger than 1000–2000 kDa, suggesting that Groucho can associate with a high molecular weight corepressor complex (Fig. 5D, first panel). Interestingly, phospho-Groucho was only detected in fractions with molecular masses of 440–670 kDa (arrow in the second panel), suggesting that the phosphorylated form of Groucho could not associate with a corepressor complex. Three different bands were detected in addition to that corresponding to Groucho (arrow in the first panel). The identity of the high molecular weight band (arrowhead) is not clear. However, the smaller molecular weight band was also detected with an antibody directed to the Groucho phosphopeptide containing the phosphoserine that is phosphorylated by DHIPK2 (Fig. 1G). Third, both DHIPK2 and mouse HIPK2 physically interact with Groucho (Fig. 4) (12). Finally, groucho and dhipk2 genetically interact in vivo (Fig. 2). Thus, our results provide the first evidence that Groucho is an in vivo target for DHIPK2. In Drosophila, only one dhipk2 gene (CG17090) exists. In mammals, however, there are three different HIPKs (25). Because a family of Groucho-related genes is present in mammals (3, 4, 42), it is conceivable that specific mammalian Groucho-related proteins can serve as targets for different HIPK phosphorylation within developmental contexts.

Although Groucho is involved in many developmental processes (13), a potential role for Groucho in eye development is less clear. Hence, it is interesting to observe the eyeless phenotype generated by overexpression of Groucho in the eye imaginal disc (Fig. 2). Because overexpression of Groucho necropises the loss-of-function phenotype of eyeless, we reasoned that Groucho might inhibit Eyeless function during the early stage of eye development. In support of this hypothesis, we demonstrated that Eyeless physically interacts with Groucho through two independent Groucho interaction domains (Fig. 3F). In particular, the transcriptional activation domain of Eyeless contains the carboxyl-terminal Groucho interaction motif, YSPW. Thus, it is likely that this physical interaction directly inhibits transcriptional activity of Eyeless (Fig. 3B). Consistent with this notion, expression of Eyeless target genes such as eya and dac was abolished (Fig. 3A). Alternatively, it is possible that Groucho might help convert Eyeless from a transcriptional activator into a repressor. These results indicate that Groucho is an essential regulator of early eye development.

Currently, it is well recognized that upon the activation of specific signaling pathways, translocation of signaling molecules into the nucleus plays an important role for the activation of target genes (22). For example, following the activation of the Wingless signaling pathway, Armadillo (β-catenin) translocates into the nucleus and interacts with a dTCF transcription factor, thereby participating in the transcriptional activation of target genes (9, 23, 47). In the absence of signals, these signal transduction pathways must be tightly regulated, and consequently, target gene expression must be maintained in a repressed state. Furthermore, given the known cross-talk among different signaling pathways, which eventually is interpreted in terms of target gene expression within the nucleus (49–51), understanding the mechanisms of how Groucho activity is regulated is crucial to understanding the nuclear events in these signaling pathways. We demonstrate here that DHIPK2 is a key player in the regulation of Groucho activity. Our results show that DHIPK2 relieves Groucho-mediated transcriptional repression by phosphorylation. The phosphorylation of Groucho induces attenuation of its protein-protein interactions with either the DNA-bound transcription factor or histone deacetylase, resulting in its dissociation from the corepressor complex. In essence, the phosphorylation status of Groucho is crucial to this process.
Regulation of Groucho Activity by DHIPK2

Combined with our preliminary data showing that mammalian HIPK2 interacts with components of either Notch or Wingless signaling (data not shown), we propose that DHIPK2 plays a pivotal role in the on-off switch of target gene repression mediated by Groucho (Fig. 6). Initially, inactive DHIPK2 tightly associates with the Groucho corepressor (Fig. 5D) and helps maintain the repressed state of target gene expression. Upon the activation of DHIPK2 by extracellular signals, activated DHIPK2 starts to phosphorylate Groucho. The phospho-Groucho then dissociates from the DNA-bound transcription factors, thereby relieving transcriptional repression (Figs. 4 and 5). At the same time, DNA-bound transcription factors together with nuclear translocated effector molecules start to recruit a coactivator complex to exert full transcriptional activation of target gene expression. Consistent with this model, our data showed that the kinase activity of DHIPK2 is essential in this process, because the kinase-inactive DHIPK2 could not overcome Groucho-mediated repression in our transient expression assays (Fig. 4A). Furthermore, our column fractionation data using embryonic nuclear extracts also support this model (i.e. in normal embryos, phospho-Groucho could not associate with a high molecular weight corepressor complex) (Fig. 5D). In fact, in the absence of Groucho DHIPK2 could enhance the Eyeless-mediated reporter gene activation (Fig. 4A).

Groucho has been shown to form a tetramer through the amino-terminal tetramerization domain, and oligomerization is required for repression in vivo (46, 52). However, it is unlikely that DHIPK2 phosphorylation of the Groucho protein affects tetramerization of the protein, since phospho-Groucho was detected in fractions larger than its monomer size (Fig. 5D). In support of this idea, phosphorylation sites were localized to the middle portion of the protein (Fig. 1) and not to the amino-terminal tetramerization domain. Probably, phosphorylation of Groucho by DHIPK2 can cause conformational changes without disrupting tetramerization, thereby inducing its dissociation from a corepressor complex.

Phosphorylation-dependent activation of transcription factors is a well known mechanism for transcriptional regulation (53–55). We have shown here that DHIPK2 phosphorylates Groucho and that this phosphorylation modulates the corepressor activity of Groucho. It is also conceivable, however, that some of the spots could arise from other kinases. Recently, it was reported that direct phosphorylation of Groucho at Ser-239 by protein kinase CK2 was important for transcription repression and inhibition of neuronal differentiation (56). However, direct phosphorylation of Groucho at Thr-308 and Ser-510 by mitogen-activated protein kinase upon epidermal growth factor receptor signaling weakens its repressor activity, attenuating Groucho-dependent transcriptional silencing by the enhancer of split proteins (57). Phosphorylation of Groucho by different protein kinases led the Groucho to function in opposite directions. Given that Groucho has involved in many signaling pathways, such as Wnt, Notch, Dpp, and Torso, it is plausible that the activity of GRO might be regulated by the combined action of these signaling molecules. Upon phosphorylation by DHIPK2, Groucho loses its corepressor activity in a manner similar to that induced by mitogen-activated protein kinase action but opposite to that induced by CK2 action. Thus, it would be very interesting to study the functional synergisms between DHIPK2 and mitogen-activated protein kinase and the functional countering between DHIPK2 and CK2. In addition, combined action of calmodulin kinase and poly(ADP-ribose) polymerase 1, which is a component of Groucho/TLE1 corepressor complex, also resulted in dismissal of the corepressor complexes and transcriptional activation of the neurogenic program (58). Taken together, these results indicate that Groucho/TLE corepressor complexes are integrators of various signaling pathways, and the repressed or derepressed state of Groucho target genes is strictly regulated by post-translational modifications of Groucho/TLE complexes through various signaling molecules. Given the fact that HIPK2 interacts with various transcription factors (25, 59–61) and HIPK2 plays a role in Wnt-induced Myb degradation in hematopoietic cells that involves TAK and NLK kinases (62), it is likely that a mammalian HIPK2 also plays a similar role during the signal-dependent transcriptional switch from repression to activation of target gene expression in the mammalian system. Thus, it will be interesting to investigate whether signaling molecules such as the Notch intracellular domain and β-catenin that are translocated into the nucleus upon the activation of the Notch and Wnt signaling pathways, respectively, can activate HIPK2.

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