Yin Yang 1 Physically Interacts with Hoxa11 and Represses Hoxa11-dependent Transcription*

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Yin Yang 1 (YY1) plays an indispensable role in embryonic development. YY1 contains an evolutionarily conserved, 22-amino acid segment, the PHO homology region (PHR), which is located within its central domain (spacer) and has been shown previously to participate in the recruitment of Polycomb group of proteins and in YY1-mediated transcription. In this report, we show that the PHR physically interacts with several Abd-B-type Hox proteins. Although ectopic expression of Hoxa11 enhanced target promoter activity, overexpression of YY1 repressed this effect, which was abrogated by YY1 siRNA and the histone deacetylase inhibitor trichostatin A. We have further demonstrated that this suppression effect was the result of YY1-dependent recruitment of HDAC2 to the Hoxa11 target promoter. Taken together, our findings show that YY1 represses Hoxa11-dependent transcription via interactions with the Hox proteins and HDAC recruitment, providing a link between an Abd-type Hox protein and a Polycomb group protein at the level of direct protein-protein interactions. These findings not only provide a novel insight into YY1 function but also identify a new regulation of homeotic protein-mediated transcriptional regulation in general.

Mammalian Yin Yang 1 (YY1) is a transcription factor involved in regulating the expression of numerous genes important for cell proliferation and differentiation (1–3). YY1 is ubiquitously expressed and conserved from Drosophila and Xenopus laevis to mammals (4, 5). YY1-deficient embryos die around the time of implantation, suggesting that YY1 plays an indispensable role in embryonic development (6).

Polycomb group (PcG) proteins are transcriptional regulators essential for the establishment and maintenance of the transcriptionally silenced state of homeotic gene expression (7). Genetic evidence suggests that the YY1 ortholog in Drosophila, pleiohomeotic (PHO), functions as a PcG protein (5). This is supported by recent findings that mammalian YY1 also functions as a PcG protein (8, 9). YY1 is the only known PcG protein containing a zinc finger domain that has the sequence-specific DNA binding capability that is conserved in PHO. It has been demonstrated that PHO binds a 17-base pair (bp) PRE sequence in the Drosophila engrailed gene (5). These early studies in Drosophila suggest an important role of YY1 in regulating homeotic gene transcription.

Although YY1 is evolutionarily conserved from Drosophila to human, the sequence homology between YY1 and PHO is limited to the DNA-binding zinc fingers and a 22-aa segment located at the central region of the protein (aa 205–226, PHO homology region (PHR)) (5). The spacer region (aa 205–295) that encompasses PHR has been shown to play a role in YY1-mediated transcriptional regulation (9). In addition, a recent study suggests that PHR may mediate physical interactions between PHO and other PcG proteins (10). A human YY1 with the spacer deleted has been shown to possess reduced transcriptional activity in vitro (11). Furthermore, removal of the spacer region in chicken DT40 cells abrogated the ability of cYY1 to support DT40 cell survival in vivo, indicating that the spacer region is required for YY1 biological functions (3). However, molecular mechanisms that underlie the spacer and PHR are incompletely understood. To address the role of the YY1 spacer region, we performed a yeast two-hybrid assay using the spacer region as bait. We identified novel physical interactions between YY1 and the Abd-B-type Hox proteins and showed that the physical interactions are important for YY1 to negatively regulate Hox protein-dependent transcription in a manner that is dependent on the recruitment of histone deacetylases. Taken together, these findings suggest that YY1 not only functions as a PcG protein to directly repress Hox gene transcription but also participates in negatively regulating transcription mediated by the products of the Hox genes, i.e. via direct physical interactions of the homeodomain proteins.

MATERIALS AND METHODS

Yeast Two-hybrid Assay—The Sall-BglII cDNA fragment encoding amino acids 205–226 of human yy1 was cloned downstream of the GAL4 DNA-binding domain into pPC98 vector and used as bait. An 8.5-day mouse embryonic cDNA library was produced using pPC86 vector (Resgen, Invitrogen). The yeast strain MaV103 (MATa, gal4, gal80, leu2, trp1, his3, SPAL10:URA3) (12) was co-transformed with pPC98/hYY1 (205–226) and the cDNA library plasmids. Transformants were plated on synthetic complete media plates lacking histidine, leucine, and tryptophan in the presence of 12.5 μM 3-amino-triazole. It produced 5.8 × 106 transformation efficiency.
An X-gal filter assay was performed after the yeast two-hybrid assay for confirmation. Yeast colonies from minimal plates (−Leu, −Trp, −His) were lifted onto nitrocellulose membranes, immersed in liquid nitrogen for 5 s, and then transferred onto Whatman filter papers saturated with 3 ml of Z-buffer (100 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM MgSO4, 40 mM β-mercaptoethanol) containing 1 mg/ml X-gal. The color of the colonies was verified after putting the membranes at 30 °C for 1 h.

Plasmids—The pPC98/PHR construct was generated in our laboratory. pBluescript/Hoxa11 was courteously offered by Steve Potter (13). The reporter construct (pGL3/promoter) containing triple Hox binding sequences (HBS) was a gift from Lingfei Luo (14). pcDNA3/FLAG-Hoxa11, pBluescript/Hoxa11 (Δaa 229–314) and pGST/Hoxa11 were constructed by PCR via pBluescript/Hoxa11. His-YY1 and pBS/U6-YY1 siRNA were reported previously (3). pcDNA3/HA-YY1 (Δaa 229–314) and pGST/YY1 (ΔPHR) were generated by PCR using pCMV/HA-YY1 as a template.

Cell Culture, Transfection, GST Pulldown, Immunoblot, and Immunoprecipitation—F9 cells and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. F9 cells were induced to differentiate by retinoic acid for 4 days. HeLa cells were transfected using Lipofectamine 2000 (Invitrogen), and immunoprecipitation was achieved as described previously (15). For GST binding assays, in vitro translated Hoxa11 and Hoxa11 lacking the homeodomain mutant products were allowed to interact with either GST or GST-YY1. The procedure for GST pulldown assays was described previously (16). Anti-Hoxa11 antibodies were generously provided by Daniela Bomgardner (17), and anti-HA (H.10) and anti-FLAG (F3165) antibodies were purchased from Babco and Sigma. Anti-YY1 (H414), anti-HDAC1 (H-51), and anti-HDAC2 (H-54) antibodies were from Santa Cruz Biotechnology.

Electrophoretic Mobility Shift Assay—Probes containing Hox and non-Hox consensus binding sites were used in this study.
Yin Yang 1 Interaction with Hoxa11

Yin Yang 1 (YY1) enhances Hoxa11 DNA binding activity. An electrophoretic mobility shift assay was performed with fusion proteins and an oligonucleotide containing a consensus core (TTAC) HBS. Binding of Hoxa11 to HBS was specific, as unlabeled HBS probe specifically abrogated its binding (lanes 5 and 7). A supershifted band was observed following the addition of Hoxa11 antibodies (lane 6). Increasing amounts of YY1 or YY1 (ΔPHR) protein were incubated with Hoxa11. A representative gel of three experiments is shown. The results indicate that YY1 acts as a binding partner of Hoxa11; increasing the amount of YY1 strongly enhanced Hoxa11 binding to HBS, whereas mutant YY1 (ΔPHR) only enhanced Hoxa11 binding slightly (lanes 8–11).

RESULTS

YY1 Interacts with Abd-B-type Hox Proteins—Human YY1 contains a 22-amino acid fragment (aa 205–226) in the spacer region (aa 205–295) that is >80% conserved in Drosophila PHO (5). To better characterize the function of the PHR in embryogenesis, we performed a yeast two-hybrid screen of an 8.5 day post coitum mouse embryo library. Amino acids 205–226 (PHR) of YY1 was used as bait (Fig. 1A). We selected color changes via X-gal filter assays. Three independent cDNAs were identified, and they encoded portions of the homeodomain proteins with a high degree of homology, i.e. Hoxa9, Hoxd9, and Hoxa11 (19, 20). These proteins are closely related to the products of the abdominal-B (Abd-B) HOM-C genes in Drosophila, which are situated at the 5′-end of the mammalian Hox clusters. Overlapping expression of these genes was found in developing limbs, indicating their specific regional identity (21, 22).

YY1 PHR Binds Hoxa11 Homeobox Domain in Vitro and in Vivo—To confirm the yeast two-hybrid results and to characterize the Hoxa11 interaction domain of YY1, we carried out a GST binding assay using in vitro translated products, full-length Hoxa11 and Hoxa11 homeodomain (ΔHD) deletion mutant (Fig. 1B). Comparable amounts of GST and GST-YY1 were used in the assays (data not shown). As shown in Fig. 1C, only full-length Hoxa11 directly interacted with YY1 (lane 3) whereas Hoxa11 ΔHD was unable to bind (lane 6), indicating that the DNA binding domain of Hoxa11 is essential for the direct interaction with YY1.

In co-immunoprecipitation (co-IP) experiments, FLAG-tagged Hoxa11 was co-transfected with HA-tagged YY1 protein into HeLa cells. Cell lysates were immunoprecipitated with
an anti-YY1 antibody. As shown in Fig. 1, YY1 interacted with Hoxa11 and Western blotted with an anti-HA antibody. Inhibition of Histone Deacetylase Activity and Depletion YY1 Abrogated the Repression of YY1 to Hoxa11-mediated Transcription—It has been shown that YY1 recruits histone modifiers to mediate transcriptional regulation, and numerous studies have revealed a functional significance of YY1 associ-
with HDACs (25–27). Therefore, we hypothesized that the repression effect observed above might be because of YY1 recruitment of HDACs. To investigate this hypothesis, the histone deacetylase inhibitor TSA was added to HeLa cells co-transfected with Hoxa11 and the HBS-Luc reporter. Significantly, compared with the controls, Hoxa11 activated promoter activity in the presence of TSA by 10-fold (Fig. 4A, lane 5), indicating that inhibition of HDAC activities reversed the suppressive effect of the endogenous YY1. Consistent with the observation that YY1 mediated Hoxa11-dependent transcription, knockdown of YY1 by siRNA in HeLa cells also enhanced Hoxa11 reporter activity (Fig. 4A, lane 4). In contrast, control experiments using the U6 siRNA vector showed no effect on reporter activity (Fig. 4A, lane 3). Thus, these findings suggest that YY1 represses Hoxa11-mediated transcription by recruiting HDACs.

**YY1 Is Required for Hoxa11 to Recruit HDAC2**—The TSA experiments described above suggest that HDACs might be associated physically with the YY1-Hoxa11 complex. To address this possibility, we transfected FLAG-Hoxa11 into HeLa cells and immunoprecipitated Hoxa11 by the FLAG antibodies followed by Western blotting to detect the presence of HDACs. As shown in Fig. 5, the FLAG antibodies co-immunoprecipitated HDAC2 from cells expressing FLAG-Hoxa11 but not from HeLa cells without FLAG-Hoxa11 expression (Fig. 5, compare lane 2 with lane 1). Strikingly, this interaction is dependent on YY1, as the Hoxa11-HDAC2 interaction was specifically disrupted by YY1 depletion (Fig. 5, lane 4). Unlike HDAC2, we found that Hoxa11 interacted with HDAC1 in the absence of YY1 (Fig. 5, lane 4). Taken together, these findings suggest that YY1 may serve as a bridge for the recruitment of HDAC2 for repression of Hoxa11-dependent transcription.

**DISCUSSION**

YY1 is essential for cell proliferation and differentiation and has been shown to play an indispensable role in embryonic development (1–3). YY1 is a PcG protein (8), and the PHO homology region (PHR, aa 205–226) has been shown to interact with other PcG proteins such as E(z) (10). A main function of the PcG proteins is to mediate repression of Hox gene transcription during development (28, 29). Consistently, YY1 has been shown to repress homeotic genes during development (8, 9). In this report, we show that PHR also mediates YY1 interactions with the products of the Abd-B-type Hox genes. This interaction results in the enhancement of Hoxa11 DNA binding activity and repression of Hoxa11-mediated transcription. Importantly, YY1 represses Hoxa11-mediated downstream target gene transcription in a manner that is dependent on its physical interactions with Hoxa11 and the recruitment of HDACs. Our findings suggest that YY1 as a PcG protein not only directly represses Hox gene transcription but also inhibits the transcriptional activities of the Hox gene products such as Hoxa11. This
YY1 was co-transfected with Hoxa11, it failed to suppress HBS-Luc reporter expression, consistent with the idea that recruitment of HDACs is important for repression (data not shown). RNA interference (RNAi) knockdown of YY1 did not result in the same level of derepression of the target gene as it did in the TSA treatment. It is possible that this may be due to an incomplete knockdown of YY1 or an involvement of additional histone deacetylases such as HDAC1 in the regulation.

The Abd-B-type Hox genes are situated at the 5′-end of the Hox clusters. Only one Abd-B gene is found in Drosophila, but a total of 16 Abd-B-type genes have been discovered in mammals (35, 36). The 5′-end of Hox genes is generally expressed in the hind limb bud (37). These genes exhibit overlapping domains of expression during limbs development, consistent with their key roles in regional identity specification (21). Hoxa11 has been shown to be expressed in the developing limbs and caudal body (13). Interestingly, elevated YY1 mRNA expression has been found in the limb bud and tail tip region as well (6). Given the findings reported in our current study, it is possible that YY1 and Hox proteins coordinate to regulate gene expression during embryogenesis.

The Hox-YY1 interaction may also be involved in regulating cell survival and/or apoptosis. Several lines of evidence indicate that Hox function is linked to apoptosis. For instance, HoxA5 has been shown to regulate expression of p53, a tumor suppressor gene, in breast cancer tissue (38). Miguel-Aliaga and Thor (39) have shown that neuronal segmental specificity is mediated by differential expression of the Abd-B Hox genes, which protect pioneer neurons from apoptosis via repression of the two RHG motif cell death activators, rpr and grim. Significantly, mammalian YY1 has also been shown to be a negative regulator of p53 recently (3, 40, 41). Removal of the PHR in chicken YY1 abrogated the ability of cYY1 to rescue cells lacking endogenous cYY1, suggesting that YY1 PHR is necessary for YY1 to support cell survival. Taken together, these findings suggest that YY1 may regulate important developmental processes as well as cell survival, in part by regulating Hox protein activities via protein-protein interactions.

Previous studies have shown that YY1 is required for Ezh2 binding to the silenced state of muscle specific genes (42) and that YY1 interacts with the EED protein in the EED-EZH human homolog PcG complex (43). These findings implicate YY1 in epigenetic regulation, specifically in mediating Hox gene repression. Importantly, our study has identified a new relationship between PcG (YY1) and Hox proteins at the level of direct protein-protein interaction. We speculate that other Hox proteins such as Hoxa9 and Hoxd9 may also be regulated by YY1 via a similar mechanism. Altogether, our findings suggest that YY1 may participate in the regulation of embryonic development in part by modulating the activity of the Hox proteins. These findings provide a new insight into YY1 function and offer a potential mechanism for the critical role of YY1 in mouse embryonic development.
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