The Homeodomain Protein NK-3 Recruits Groucho and a Histone Deacetylase Complex to Repress Transcription*

Communication

(Received for publication, August 23, 1999, and in revised form, September 20, 1999)

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Transcriptional repression by sequence-specific DNA binding factors is mediated by the recruitment of a corepressor complex to the promoter region. The NK-3 homeodomain protein is a transcriptional repressor that recruits the nuclear protein kinase, homeodomain interacting protein kinase 2 (HIPK2). Here we show that HIPK2 is a component of a corepressor complex containing Groucho and a histone deacetylase complex. Groucho, like HIPK2, acts as a corepressor for NK-3 and binds to NK-3 and HIPK2. Moreover, HIPK2 appears to regulate the corepressor activity of Groucho. Transcriptional repression by NK-3 and Groucho is relieved by the histone deacetylase inhibitor trichostatin A, and both NK-3 and Groucho directly interact with the histone deacetylase HDAC1 that is associated with mSin3A in vivo. Recruitment of the histone deacetylase complex by NK-3 decreases the acetylated histones that are associated with the target gene promoter. These results indicate that NK-3 represses transcription by recruiting a complex containing Groucho and a histone deacetylase complex that leads to histone modification on chromatin and suggest that HIPK2 may play a regulatory role in the corepressor complex formation.

The critical components of a corepressor complex (1, 2) are histone deacetylases that deacetylate core histones on chromatin (3–5) and corepressors that connect DNA-binding factors to the histone deacetylase complex (6–11). Among corepressors, it has been well established that Groucho proteins can serve as corepressors for several distinct types of active repressors such as the Hairy-related and Runt domain proteins (12–14). However, it has not been demonstrated whether Groucho, like other corepressors, can interact with the histone deacetylase complex (12).

We have previously shown that the NK-3 homeodomain protein (15), which belongs to the NK-2 class that includes a large number of vertebrate homeodomain transcription factors (16), is a transcriptional repressor and that the nuclear protein kinase HIPK2† can act as a corepressor for NK-3 (17). During the course of investigating the function of HIPK2, we noticed that HIPK2 could interact with Groucho. Because it was unknown whether Groucho is involved in the NK-3-mediated transcriptional repression, it was of interest to examine whether Groucho can act as a corepressor for NK-3. Here we show that the NK-3 homeodomain transcription factor recruits the Groucho corepressor, HIPK2, and a histone deacetylase complex to repress transcription.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Transfections—Cell transfections (5 × 10⁵ cells per transfection) and CAT assays were performed as described previously (18). The NK3CAT reporter was constructed by inserting a XhoI-DNA fragment (1.6 kilobase), which includes both the 5′ upstream region containing NK-3 autorepression sites (NK3AR), and the promoter region of NK-3 into the SacI site of the pCAT-basic vector (Promega). For the constructions of the FLAG-, GFP-, and Myc-tagged expression vectors, each cDNA was cloned into the CMV-Tag1 (Stratagene), pEGFP-C2 (CLONTECH), and pCS3 + MT vectors, respectively. For generating the mutant expression vector, PCR-based mutagenesis was employed.

Chromatin Immunoprecipitation and Western Blot Analysis—HeLa cells were transfected with the indicated plasmids, and nuclear extracts (1 mg of nuclear proteins) were immunoprecipitated with either anti-FLAG antibody (Sigma) or an anti-GFP antibody (CLONTECH), followed by Western blot with either an anti-Myc antibody (Invitrogen) or anti-mSin3A (K20, Santa Cruz Biotechnology) as described (9).

In Vitro Pull-down Assays—Various NK-3 and Groucho cDNAs were subcloned into the pSPUTK vector and subjected to in vitro transcription using the TNT coupled reticulocyte lysate system (Promega). Pull-down assays were performed by incubating equal amounts of GST or the indicated GST-NK3, GST-GRO, GST-HIPK2, and GST-HDAC1 fusion proteins immobilized onto glutathione-Sepharose beads with various in vitro translated, ³²P-labeled truncated forms of Groucho and NK-3 proteins as described (17). Expression and purification of the fusion proteins were performed as described previously (18).

Histone Deacetylase (HDAC) Assays—HDAC assays were performed using ³¹H-acetate-labeled histones (13,000 dpm/reaction) as described (9, 19). Samples were assayed in duplicate in the presence or absence of 0.6 mM trichostatin A.

Chromatin Immunoprecipitation (CHIP) Assays—CHIP assays were performed using a CHIP assay kit (Upstate Biotechnology) as described (5, 20). Cells (2 × 10⁹) were transfected with indicated plasmids (5 µg of reporter plasmids and 10 µg of expression vector) and fixed with 1% formaldehyde for 10 min before harvesting. The cross-linked chromatin were immunoprecipitated with antibodies to the acetylated histones H3 and H4. Total DNA (50 µl) recovered from the immunoprecipitates was subjected to quantitative PCR (25 cycles of 30 s at 94 °C, 30 s at 65 °C, 45 s at 72 °C) using specific primers (P1, 5′-ATTTAGGG-TGACACATAGA-GTGC-3′; P2, 5′-GAAAGGTGTCAGTTAGGGTG-3′) and 2 µl of template DNA. The band intensities of the PCR products were measured with the Stratagene Eagle Eye II image analysis program.

Gel Filtration—Three hundred µl of nuclear extract (10 mg/ml) was run on a Superose 6-gel filtration column (10 × 300 mm, Amersham Pharmacia Biotech) equilibrated with Buffer B containing 50 mM Hepes (pH 7.9), 150 mM NaCl, 1 mM EDTA, and 0.1% Nonidet P-40. After sample injection, fractions of 300 µl were collected (flow rate, 0.3 ml/min). An aliquot (15 µl) of every fraction was subjected to Western blot analysis. The following proteins were used for calibration: thyroglobulin (670 kDa), ferritin (440 kDa), aldolase (160 kDa), and albumin (68 kDa).

The abbreviations used are: HIPK2, homeodomain interacting protein kinase 2; aa, amino acid(s); CAT, chloramphenicol acetyltransferase; CHIP, chromatin immunoprecipitation; GFP, green fluorescent protein; GRO, Groucho; GST, glutathione S-transferase; HDAC, histone deacetylase; PCR, polymerase chain reaction; TSA, trichostatin A.

* This work was supported by a grant from the NHLBI, National Institutes of Health, Intramural Program (to Y. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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RESULTS AND DISCUSSION

We show that Groucho acts as a corepressor for NK-3 (Fig. 1). Repressor activity of NK-3 on the native NK-3 promoter (NK3CAT), which contains autorepression sites, was enhanced by Groucho (Fig. 1A, lanes 2 and 5). This effect was not seen using NK3NQ, which has a mutation within the homeodomain and cannot bind to target sequences (Fig. 1A, lanes 3 and 6). Groucho also enhanced the repressor activity of GAL4-NK3, in which NK-3 was tethered to a heterologous GAL4 DNA binding domain, on a synthetic promoter (G5EnXCAT) (Fig. 1A, lanes 9–11). In contrast, GAL4-NK3aHD, which does not show any repressor activity alone (lane 12) and cannot interact with Groucho (see below) because of deletion of the homeodomain, did not respond to Groucho (Fig. 1A, lanes 13–15). Thus, these results indicate that NK-3-mediated transcriptional repression, either on the native promoter or on a synthetic promoter, is greatly augmented by Groucho and suggest that the Groucho-enhanced repressor activity of NK-3 occurs through protein–protein interaction.

To test whether NK-3 can recruit Groucho in vivo, nuclear extracts from cells transfected with expression vectors for FLAG-tagged NK-3 (FLAG-NK3) and Myc-tagged Groucho (Myc-GRO) were subjected to immunoprecipitation with an anti-FLAG antibody and were analyzed by Western blot with an anti-Myc antibody (Fig. 1B). Indeed, Groucho was immunoprecipitated only from cells cotransfected with NK-3 and Groucho (Fig. 1B, lane 6), suggesting that Groucho is associated with NK-3 in vivo. In addition, the GFP-GRO(WD) fusion protein formed a complex with HDAC1 (see Fig. 1D, +, weak binding; ++, strong binding; +++, very weak binding; −, no binding).

![Figure 1](image1.png)  
**Fig. 1.** Groucho acts as a corepressor for NK-3. A, Groucho enhances transcriptional repressor activity of NK-3. The effects of Groucho on the repressor activity of either NK-3 (lanes 1–6, 2 μg/ transfection) or GAL4-NK3 (lanes 7–15, 0.1 μg/ transfection) were measured by transient expression assays in cultured cells. The normalized CAT activity obtained from transfection with a reporter and an empty vector (lanes 1–6, pRC-CMV; lanes 7–15, pSG424) was divided by the corresponding value obtained with a test expression vector, and fold repression is shown. The amounts of Groucho expression vectors used are as follows: 1 μg (lanes 4–6, 10, and 14), 0.5 μg (lanes 9 and 13), and 2 μg (lanes 11 and 15)/transfection, respectively. B, recruitment of Groucho in vivo. C, in vivo escort assay. Cells were transfected with GFP-GRO(WD) in the absence (left) or presence (right) of NK-3, and GFP signals were detected. D, interaction of NK-3 with Groucho in vitro. GST pull-down assays were performed with in vitro translated NK-3 and GST-Groucho (aa 1–719). GST, negative control; GRO, pull-down assays with a GST-Groucho protein. E, GST pull-down assays with in vitro translated Groucho and GST-NK-3 (aa 1–382).

![Figure 2](image2.png)  
**Fig. 2.** Association of transcriptional repression by NK-3 and Groucho with histone deacetylase activity. A, the histone deacetylase inhibitor, TSA, relieves repression by NK-3 and Groucho. Cells were transfected with the indicated reporter (1 μg/ transfection) and expression vectors in the presence (+) or absence (−) of TSA (0.3 μM). Fold repression is calculated as in Fig. 1A. Expression vectors used are as follows (μg/ transfection): NK-3 (lanes 2, 5, 6, and 7, 0.5 μg; lane 3, 1 μg; lane 4, 2 μg); GRO (lanes 5, 6, and 7, 0.5, 1, and 2 μg, respectively); GAL4-NK3 (lanes 9, 10, and 11, 0.25, 0.5, and 1 μg, respectively); GAL4-GRO (lanes 12, 13, and 14, 0.25, 0.5, and 1 μg, respectively). B, mapping the HDAC interaction domain of NK-3. A GST-HDAC1 fusion protein (aa 1–489) was input; G and H, pull-down assays with a GST control or a GST-HDAC1 protein, respectively. Constructs used for in vitro translation are shown under the schematic diagram of Groucho. Results of NK3-binding are also indicated (see Fig. 1E). D, mapping the HDAC interaction domain of NK-3. Pull-down assays were performed with in vitro translated NK-3 proteins and GST-HDAC1. Results of Groucho binding are also indicated (see Fig. 1D). +, weak binding; ++, strong binding; +++, very weak binding; −, no binding.
nucleus when cotransfected with NK-3 (Fig. 1C, right panel). This result indicates that GFP-GRO(WD) can associate with NK-3 in vivo in the absence of DNA and that this interaction helps its translocation into the nucleus. Using a GST-Groucho protein and various in vitro translated NK-3 proteins, pull-down assays were performed to test direct interaction between the two proteins. The full-length NK-3 can strongly bind to Groucho (Fig. 1D, lane 3). Deletion of either the C terminus (aa 302–382) or the N-terminal half (aa 1–162) of NK-3 does not affect its binding activity to Groucho (Fig. 1D, lanes 6 and 15). However, further deletion of the homeodomain from these constructs abolished (Fig. 1D, lane 9) or weakened (Fig. 1D, lane 18) the binding activity to Groucho, demonstrating that the homeodomain of NK-3 is important for strong interaction with Groucho. Converse experiments using in vitro translated Groucho proteins confirmed direct binding of Groucho to GST-NK3 (Fig. 1E, lane 3). For the interaction of Groucho with NK-3, both the WD 40-repeat domain located in the C terminus, which was shown to function as a protein-protein interaction domain (21), and the N terminus of Groucho, which is involved in tetramerization of Groucho (22), are required (Fig. 1E, lanes 9 and 15). Taken together, these results indicate that NK-3 directly interacts with Groucho, strongly suggesting that Groucho acts as a corepressor for the NK-3 homeodomain transcription factor. Because corepressors, which are recruited to target promoters, associate with the mSin3-histone deacetylase complex (6–11), we then asked whether histone deacetylase activities are involved in NK-3 and Groucho-mediated repression. Transfected cells were treated with the histone deacetylase inhibitor trichostatin A (TSA) (19), and repressor activities of NK-3 and Groucho were compared with those from untreated cells. TSA treatment alleviated repressor activities of NK-3 on the NK-3 promoter (Fig. 2A, lanes 1–7). Also, TSA treatment was effective in relieving the repressor activities of GALA-NK3 and GALA-GRO (Fig. 2A, lanes 8–14). These results suggest that the histone deacetylase activities are associated with the repressor activities of NK-3 and the Groucho corepressor. Indeed, immunoprecipitation experiments showed that both NK-3 and Groucho associate with the histone deacetylase HDAC1 in vivo (Fig. 2B, lanes 8 and 16). Groucho directly interacts with HDAC1 in vitro (Fig. 2C, lane 3). Interestingly, the N-terminal region of Groucho is important for strong binding to HDAC1 (Fig. 2C, lane 9), which coincides with the fact that the N-terminal region can act as an active repression domain when tethered to DNA-binding proteins (22, 23). We found that NK-3 also binds...
directly to HDAC1 (Fig. 2D) and that the interaction domains with HDAC1 are similar to those of the Groucho-binding domains. Furthermore, the immunoprecipitated proteins showed histone deacetylase activity in vitro, which was inhibited by the presence of TSA (Fig. 3A). Consistent with the well-established results seen in the mSin3-histone deacetylase complex (6–11), mSin3A was also present in the coprecipitated proteins (Fig. 3B). Taken together, these results indicate that NK-3 recruits a corepressor complex containing Groucho, mSin3A, and histone deacetylase to repress transcription.

Recruitment of the histone deacetylase complex to the target promoter generates a highly localized domain of repressed chromatin (3–5). Hence, the deacetylase complex recruited by NK-3 and Groucho may modify chromatin. To test this idea, CHIP assays were employed (Fig. 3C) (20). Two different reporters were cotransfected into cells with either GAL4-NK3 or GAL4. G5EnXCAT contains an SV40 enhancer and GAL4 binding sites, and responds to GAL4 proteins. The EnXCAT, lacking GAL4 binding sites, does not respond to GAL4 protein but forms active chromatin; hence this reporter was used as an internal control for the CHIP assays. The precipitated DNAs, following immunoprecipitation of chromatin, was used as an internal control for the CHIP assays. The precipitated DNAs, following immunoprecipitation of chromatin, were subjected to a quantitative PCR with primers (Fig. 3C, P1 and P2), and the intensities of the amplified bands (G5, 404 bp from G5EnXCAT; G0, 281 bp from EnXCAT) were measured. We found that the ratio (G5/G0) of band intensity amplified from the chromatins of cells transfected with GAL4-NK3 is remarkably reduced (Fig. 3C, lanes 4, 5, and 7) when compared with that of cells transfected with GAL4 (Fig. 3C, lanes 2, 3, and 6). These results indicate that recruitment of the histone deacetylase complex to the target promoter region by GAL4-NK3 decreased the acetylated histones H3 and H4, suggesting that this modification of histones may generate a domain of repressed chromatin in vivo.

We have previously shown that HIPK2 can act as a corepressor for NK-3 (17). Hence, we wondered whether HIPK2 was a component of the corepressor complex described above. To test this idea, nuclear extracts from transfected cells were subjected to Superose 6-gel filtration column, and fractions were analyzed by Western blot. As shown in Fig. 4A, HIPK2, Groucho, and NK-3 were detected in fractions larger than their monomer sizes (peak fractions 46, 42, and 52, respectively). In addition, all three proteins were also eluted ahead of a 670-kDa marker, molecular mass 1000-2000 kDa, suggesting that the three proteins are associated with other nuclear proteins. Endogenous mSin3A, HDAC1, and RbAp48 showed a broad elution profile (Fig. 4A), indicating that they participate in different complexes. Groucho and HIPK2 are immunoprecipitated with NK-3 (Fig. 4B). Also, mSin3A and HDAC1 were detected in the same immunoprecipitate, which is consistent with our results described above (Figs. 2 and 3) and suggests that they associate with each other to form a corepressor complex. A separate series of experiments with nuclear extracts prepared from cells cotransfected with HIPK2 together with either NK-3 (Fig. 4C) or Groucho (Fig. 4D) revealed that HIPK2 associates with NK-3 and Groucho in vitro. Two HIPK2 bands were detected (Fig. 4C and D, lanes 2 and 3), one of which is presumably the modified form of HIPK2. Interestingly, both NK-3 and Groucho recruit the faster migrating HIPK2 band (Fig. 4, C and D, lane 6, arrows). In addition, HIPK2 binds directly to Groucho and NK-3 in vitro (Fig. 4E). Taken together, these results indicate that HIPK2 directly interacts with NK-3 and Groucho and suggest that HIPK2 is a component of the corepressor complex recruited by NK-3.

Finally, we investigated the effect of HIPK2 on the corepressor activity of Groucho using cotransfection assays with either wild type HIPK2 or kinase-inactive HIPK2KR (Fig. 4F). We used GAL4-NK3 (lanes 2–7) and G5EnXCAT as reporters, and GAL4-NK3HD was used as a control (lanes 8–13). We found that, in the absence of Groucho, the wild type HIPK2 enhanced the repressor activity of GAL4-NK3 (Fig. 4F, lane 3). In the presence of Groucho, however, wild type HIPK2 did not enhance the corepressor activity of Groucho further (Fig. 4F, lane 6). Instead, wild type HIPK2 seemed to relieve the corepressor activity of Groucho further (Fig. 4F, lanes 5 and 6). Interestingly, the kinase inactive mutant HIPK2 (HIPK2KR) can enhance the corepressor activity of Groucho (Fig. 4F, lane 7). These results indicate that the corepressor activity of Groucho is regulated by HIPK2 and suggest that the kinase activity of HIPK2 is involved in this regulation.

In summary, our results indicate that the NK-3 homeodomain protein recruits a corepressor complex containing Groucho, HIPK2, and a histone deacetylase complex to repress transcription. In light of our results, we propose that a global mechanism of repression, deacetylation of histones, is involved in transcriptional repression by the homeodomain transcription factor. We have recently demonstrated that HIPK2 is covalently modified by the ubiquitin-like protein, SUMO-1, and that SUMO-1-modification of HIPK2 correlates with its localization to nuclear dots (24). In this regard, it is of interest to speculate that HIPK2 may play a regulatory role in the formation of a corepressor complex.

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