A novel family of cofactors that differentially interact with homeoproteins have been identified via a yeast two-hybrid screen. The proteins contain a conserved protein kinase domain that is separated from a domain that interacts with homeoproteins and hence are termed homeodomain-interacting protein kinases (HIPKs): HIPK1, HIPK2, and HIPK3. We show that HIPKs are nuclear kinases using GFP-HIPK fusion constructs. The DNA binding activity of the NK-3 homeoprotein is greatly enhanced by HIPK2, but this effect is independent of its phosphorylation by HIPK2. In cultured cells, HIPKs localize to nuclear speckles and potentiate the repressor activities of NK homeoproteins. The co-repressor activity of HIPKs depends on both its homeodomain interaction domain and a co-repressor domain that maps to the N terminus. Thus, HIPKs represent a hitherto undescribed family of co-repressors for homeodomain transcription factors.

Homeobox genes play important roles in diverse developmental processes such as pattern formation, organogenesis, and the determination of cell fate in various organisms (1). These homeobox genes encode sequence-specific DNA-binding proteins that act as both transcriptional activators and repressors that control the expression of target genes in a temporally and spatially regulated manner (1, 2). Homeodomain transcription factors have been shown to interact with cofactors that help to direct different homeoproteins to different target genes in vivo (3). In most cases the cofactors are transcription factors that contain their own DNA-binding domains (4–9). To date a cofactor for a homeoprotein that shows enzymatic activity has not been reported.

The NK homeobox genes (10, 11), especially the NK-2 family of homeobox genes, were shown to have important functions during embryonic development and organogenesis including development of the heart (reviewed in Ref. 12). The recent discovery that mutations in the gene encoding the NKX2-5 homeodomain transcription factor were found to cause nonsyndromic, human congenital heart disease provides the first evidence that NKX2-5, a member of the NK-2 family of homeobox genes, is also involved in human disease (13). Cofactors for NK homeoproteins are totally unknown. In an effort to identify genes encoding proteins that interact with NK homeodomain transcription factors and to evaluate their contribution to the functional specificity of the NK homeoproteins, a yeast two-hybrid screen of mouse embryonic matchmaker cDNA libraries (days 7 and 11) was performed using a portion of the mouse Nkx-1.2 homeoprotein as bait. Here, we report the cloning of potential cofactors that differentially interact with homeoproteins. Further, we show that these HIPKs constitute a novel family of nuclear protein kinases that potentiate the transcriptional activities of homeoproteins.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screens and cDNA Cloning**—The two-hybrid screen was performed essentially as described (14). For bait construction, a DNA fragment (0.6 kb, a.a.110–305) from Nkx-1.2 which is one of the two mouse homologues of *Drosophila* NK-1 (15, 16), was subcloned into the EcoRI site of pGB79 (CLONTECH). Approximately 1.2 × 10<sup>7</sup> transformants from mouse embryonic days 7 and 11 matchmaker cDNA libraries (CLONTECH) were screened in the H7Fc yeast strain containing the bait plasmid. β-Galactosidase activity was measured with liquid culture assays and filter lift assays. For deletion analysis of the Nkx-1.2 interaction domain in yeast, DNA fragments were amplified by PCR with specific primers and were fused in frame with the GAL4 DNA-binding domain of pGAT9. For cDNA cloning, mouse brain and heart cDNA libraries (Stratagene) were screened with radioactively labeled DNA fragments obtained from the yeast two-hybrid screen (clone 7 for Hipk1, 0.94 kb, and clone 41 for Hipk2, 1.0 kb). A total of 36 overlapping clones were sequenced and classified (16 clones for Hipk1, 16 clones for Hipk2, and 6 clones for Hipk3). The longest cDNA fragment obtained is a 4.0-kb Hipk2 cDNA, which covers nearly the full open reading frame for HIPK2. For cloning of the 5′ end of the cDNA reverse transcription-PCR was used with selected primers for HIPKs and mouse heart mRNA.

**Expression of Proteins and in Vitro Pull-down Assay**—The GST-NK homeodomain fusion proteins were expressed in *Escherichia coli* using the pGEX-5X-1 vector and purified as described (17). For the expression of HIPK2, an EcoRI DNA fragment (4.0 kb, amino acids 25–1189) was cloned into the pFastBac expression vector (Life Technologies, Inc.), and recombinant Bacmid DNAs were recovered as per the manufacturer’s protocol. His-tagged HIPK2 proteins were purified using a nickel-charged Sepharose column (Promed, Invitrogen). For pull-down assays, Hipk1 and Hipk2 clones (clone 7 and 41, respectively) obtained from the yeast two-hybrid screen were subcloned into a pSPUTK vector and subjected to in vitro translation using the TNT Coupled Reticulocyte Lysate System (Promega). Pull-down assays were performed by incubating equal amounts of GST or GST-Nkx1-2, immobilized onto glutathione-Sepharose beads, with *in vitro* translated HIPK1 and HIPK2 proteins diluted in phosphate-buffered saline. The mixtures were placed on ice for 2 h and washed five times with phosphate-buffered saline and 1% Triton X-100, and bound proteins were eluted, separated by 12% SDS-polyacrylamide gel electrophoresis, and autoradiographed.

**In Vitro Phosphorylation of Homeoproteins by HIPK2 and Gel Shift**

The abbreviations used are: HIPK, homeodomain-interacting protein kinase; kb, kilobase(s); GST, glutathione S-transferase; a.a., amino acid(s); PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; GFP, green fluorescence protein; SRS, speckle retention signal; CRD, co-repressor domain.
Assay—For testing the phosphorylation of NK homeodomain proteins, GST-NK fusion proteins (300 ng each) were incubated with HIPK2 (300 ng) at 30 °C for 2 h in kinase buffer A containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, and 1 mM dithiothreitol supplemented with 0.1 mM ATP and [γ-³²P]ATP to a final specificity of 100 μCi/μmol. Reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiographed. Gel shift assays were performed as described (17) with a ³²P-labeled DNA probe (5′-TCTGCACTTAAGCGCCACTTAAC-3′) that contains two copies of the NK-3 target sequence. To see whether phosphorylation can affect the DNA binding activity of NK-3, GST-NK-3 (300 ng) was preincubated with HIPK2 (300 ng) in kinase buffer A in the presence or in the absence of 0.1 mM ATP for 2 h at 30 °C, and aliquots (one-tenth) of the reaction mixture were used for gel shift assays.

Cell Transfections and CAT Assays—Transfections into CV-1 cells with the indicated plasmids using the calcium phosphate precipitation method, normalization of transfection efficiency, and CAT assays were performed as described (17). The G5EnXCAT reporter, which contains the SV40 enhancer between the GAL4 DNA-binding sites and the TATA box of G5BCAT reporter, produced high CAT activity in transfected cells, so that it was easier to measure the repression activity of GAL4-NK-3 or GAL4-NK-1. For the construction of HIPK2-K221R (Lys 221 replaced with Arg), HIPK2 was used as bait with specific primers (primer 2558, 5′-AATGAAATTTGTTGCCCATAGCAGCACCACCC-3′ and primer 2559, 5′-GGGGTTCTGGATGTTGAGCCCAATTCATT-TCATT-3′). The DNA fragments (1.0 kb, coding region for a.a. 1–328) of the corresponding kinase domain of the wild type HIPK2 was replaced by the same DNA fragments containing the mutation (K221R) that was obtained by PCR. Various GAL4-HIPK2 constructs were generated from the full-length GAL4-HIPK2 by deletions of specific regions (indicated as a.a. numbers) using restriction sites. 1 μg of reporter plasmid (G5EnXCAT) and 2 μg of CMV-HIPK2 were used for each transfection. The amounts of effector plasmids used for each transfection were 40 ng (GAL4-NK-3 or GAL4-NK-1) and 500 ng (GAL4-HIPK fusion constructs), respectively.

RESULTS AND DISCUSSION

Among the thirty-eight positive clones that were identified from the two-hybrid screen and sequenced, two clones (clones 7 and 41) showed significant identities in amino acid residues to each other and were further characterized. These two clones (HIPK1 and HIPK2, see below) showed a strong interaction in the yeast two-hybrid assays (Fig. 1A, lanes 3 and 5). Interactions of HIPK1 (Fig. 1B, lanes 1–4) and HIPK2 (Fig. 1B, lanes 5–8) with Nkx-1.2 were also confirmed by pull-down assays with in vitro translated ³²S-labeled HIPK1 (lane 2) and HIPK2 (lane 6). Both HIPK1 (lane 4) and HIPK2 (lane 8) showed strong interactions with the GST-Nkx-1.2 protein, whereas they did not interact with the GST control protein (lanes 3 and 7). Deletion analysis of Nkx-1.2 (Fig. 1C) revealed that the homeodomain is required (Fig. 1C, constructs D9 and D10) but is not sufficient (Fig. 1C, construct D4) for the interaction with HIPK1 and HIPK2.

Using clones 7 and 41 as probes, cDNAs from mouse brain and heart cDNA libraries were cloned, and the nucleotide sequences were determined. Surprisingly, sequence analysis revealed an open reading frame coding for a protein kinase motif (Fig. 2A). Because both genes can encode the catalytic domain of a protein kinase, we named these genes Hij1p and Hij2p (homeodomain-interacting protein kinases 1 and 2). From this screening we also cloned Hij3p, a third member of the HIPK family. Comparison of the deduced amino acid sequences revealed strong similarity in the protein kinase domains (Fig. 2, A and B). A search of the GenBank™ database revealed strong similarities with known expressed sequence tags and a nematode DNA sequence from Caenorhabditis elegans (F20B6.8) that contains a hypothetical open reading frame for a protein kinase, suggesting that HIPKs are well conserved in other species. In fact, a dendrogram analysis indicated that HIPKs constitute an unique family of protein kinases that show the closest similarity to the yeast YAK1 (43% identity in the catalytic domain) that belongs to the CMGC group (Ref. 18 and data not shown). The homeoprotein interaction domains, which were deduced from the original yeast clones, also show significant amino acid identities (Fig. 2, A and B). Interestingly, an additional amino acid sequence in the interaction domain of HIPK1 was found (Fig. 2A). As shown in Table I, HIPRs interact with other NK homeoproteins such as Drosophila NK-1, NK-3, and mouse Nkx-2.5 as well as with HoxD4 and HoxC4. Interestingly, HIPKs showed differential interactions with different homeoproteins, although the interaction domains show significant amino acid identities. Because the baits that showed interaction with HIPKs contain the homeodomain and the C terminus of the proteins (Table I), these results suggest that the homeodomains may be important for specific interactions with HIPKs. HIPKs contain a PEST sequence (19) following the interaction domain (Fig. 2, A and B). A C-terminal region enriched in tyrosine and histidine residues (Fig. 2B, the YH
domain) with unknown function is also conserved among the HIPKs.

To explore the possibility that HIPKs are nuclear kinases, the subcellular localization of HIPKs using green fluorescence protein (GFP) was determined in living cells. When CV-1 cells were transfected with GFP-HIPK2 (Fig. 2C), the fluorescence derived from GFP was preferentially detected in nuclear speckles (dots) (20), suggesting that HIPKs are nuclear protein kinases. Consistent with these results, further deletion analysis of HIPK2 revealed multiple nuclear localization signals and a nuclear speckle retention signal (SRS, a.a. 860–967) (21) that follows the interaction domain and overlaps with a PEST sequence.2 Homeoproteins are highly phosphorylated during development (22). The function of phosphorylation as well as the identity of the protein kinases are unknown. HIPKs may be a group of kinases that are responsible for the phosphorylation of homeodomain transcription factors, because they interact with a subset of NK homeodomain transcription factors. Indeed, when HIPK2 was purified following baculovirus expression, it was capable of phosphorylating NK homeoproteins in vitro (Table I). However, the function of this phosphorylation is still unknown.

FIG. 2. HIPKs constitute a novel family of nuclear protein kinases. A, amino acid sequence comparison among HIPKs. Amino acid sequences of HIPK1, HIPK2, and HIPK3 were aligned using the GCG program PILEUP. The initiator methionine amino acid is numbered as 1. Black highlighting with white characters indicates identical amino acids, and light gray-highlighted amino acids represent similarities. A dot indicates a gap. B, schematic structural diagram of HIPK1, HIPK2, and HIPK3 protein showing their similarities. Regions in HIPK1 and HIPK3 with significant similarity are aligned with HIPK2, and the percentage of amino acids similar to HIPK2 (including conservative amino acid replacement) is indicated. Numbers on top indicate amino acid residues. C, nuclear localization of GFP-HIPK2 protein. CV-1 cells were transfected with GFP or GFP-HIPK2 and visualized with GFP fluorescence 48 h after transfection.

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The interaction between the homeoprotein and HIPK2 is required for enhanced repressor activity of GAL4-NK-3 (lane 4). These results strongly suggest that interaction between the homeoprotein and HIPK2 is required for enhancement of NK-3 repressor activity and that HIPK2 can act as a co-repressor for NK-3. Consistent with this idea, HIPK2 also acts as a co-repressor for NK homeodomain transcription factors.

A. HIPK2 enhances the repressor activities of NK homeodomain proteins. The normalized CAT activity from cotransfection with reporter (G5EnXCAT) and GAL4-NK-3 was divided by the corresponding value obtained by cotransfection with the CMV-HIPK2 expression vector and effector (GAL4-NK-3) or GAL4-NK-1 and is shown as fold repression. B. The enhanced repressor activity of GAL4-NK-3 by HIPK2 depends on multiple domains of HIPK2. Cells were cotransfected with the same reporter (G5EnXCAT) and effectors (GAL4-NK-3) in the presence of mutant HIPK2 expression vectors (HIPK2-N, a.a. 1–629; HIPK2-C, a.a. 503–1189; HIPK2-K221R, the kinase inactive mutant HIPK2), and CAT activities were measured. Repression fold obtained from each construct was compared with the corresponding value obtained by the wild type HIPK2 expression vector (HIPK2-F) and is shown as relative fold repression. HIPK2-F showed 5-fold repression (regarded as 100%) compared with that obtained with transfection with GAL4-NK-3 alone. C. HIPK2 contains a strong CRD. Schematics of various GAL4-HIPK2 fusion constructs (A–Q, indicated at the left side of each construct) are shown under the schematic structural diagram of HIPK2. The normalized CAT activity from cotransfection with reporter (G5EnXCAT) and the GAL4 plasmid (pSG424) was divided by the corresponding value obtained by cotransfection with various GAL4-HIPK2 fusion plasmids and is shown as repression fold (averages of six sets of independent experiments). K→R, Lys to Arg mutation at a.a. 221.

FIG. 4. HIPK2 acts as a co-repressor for NK homeodomain transcription factors. A. HIPK2 enhances the repressor activities of NK homeodomain proteins. The normalized CAT activity from cotransfection with reporter (G5EnXCAT) and GAL4 plasmid (pSG424) was divided by the corresponding value obtained by cotransfection with the CMV-HIPK2 expression vector and effector (GAL4-NK-3) or GAL4-NK-1 and is shown as fold repression. B. The enhanced repressor activity of GAL4-NK-3 by HIPK2 depends on multiple domains of HIPK2. C. HIPK2 contains a strong CRD. Schematics of various GAL4-HIPK2 fusion constructs (A–Q, indicated at the left side of each construct) are shown under the schematic structural diagram of HIPK2. The normalized CAT activity from cotransfection with reporter (G5EnXCAT) and the GAL4 plasmid (pSG424) was divided by the corresponding value obtained by cotransfection with various GAL4-HIPK2 fusion plasmids and is shown as repression fold (averages of six sets of independent experiments). K→R, Lys to Arg mutation at a.a. 221.

**TABLE I**

**Interactions of HIPKs with homeodomain proteins**

Various coding regions (amino acid numbers) of homebox genes were subcloned into pGBT9 vector, and interactions were determined by β-galactosidase assays in yeast. In vitro phosphorylation of bacterially expressed NK homeodomain proteins by HIPK2 were tested.

| Bait       | HIPK1 | HIPK2 | HIPK3 | HIPK2 |
|------------|-------|-------|-------|-------|
| pGPT9      | −     | −     | −     | ND    |
| NKx-1.2 (110–305) | ++   | ++    | ++    | +     |
| NK-1 (1–661) | ++    | ++    | ++    | +     |
| NK-1 (1–304) | −     | −     | −     | ND    |
| NK-1 (474–661) | ++   | ++    | ++    | +     |
| NK-3 (1–382) | +     | +     | −     | ND    |
| NK-4 (267–416) | −     | −     | −     | ND    |
| NKx-2.5 (19–315) | +     | +     | −     | ND    |
| Hox/C (124–262) | −     | −     | −     | ND    |
| Hox/C (122–250) | +     | +     | −     | ND    |

**a** ++, strong interaction; +++, no interaction. **b** +, positive; −, negative; ND, not determined.

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Conversely, deletion of the C-terminal half of HIPK2, including the homeoprotein interaction domain (construct HIPK2-K, a.a. 1–629) abolished this effect (Fig. 4B, lane 2), indicating that interactions between HIPK2 and homeoproteins are important for enhancing the repressor activities of homeoproteins. A truncated form of HIPK2 (construct HIPK2-C, a.a. 503–1189), which has a deletion of the N-terminal half including the protein kinase domain, did not enhance the repressor activity of NK-3 either (Fig. 4B, lane 3). These results imply that the enhancement of repressor activity of homeoproteins by HIPK2 is also conferred by domains within the N-terminal half of the HIPK2 (co-repressor domain, see below) in addition to the homeoprotein interaction domain in the C-terminal half.

To investigate the possible role of the protein kinase activity of HIPK2 in enhancing the repressor activity of NK-3, we generated a kinase inactive mutant form of HIPK2 (construct HIPK2-K221R) that has a mutation in an invariant amino acid (Lys to Arg at position 221) in the active site of the protein kinase domain (subdomain II) and blocks catalytic activity based on studies of other protein kinases including mammalian mitogen-activated protein kinases (18, 23). In fact, the repressor activity of NK-3 was reduced in cells cotransfected with this mutant, HIPK2-K221R, when compared with that of the wild type HIPK2 (50% reduction; Fig. 4B, lane 4). These results suggest that the protein kinase activity is at least partly required for the full co-repressor activity of HIPK2.

If HIPKs can act as co-repressors, then they should have co-repressor domain(s) that show repressor activity when tethered to DNA. To assess this possibility, GAL4-HIPK2 fusion constructs were generated, and their repressor activities were measured (Fig. 4C). As expected from the results described above (Fig. 4A and B), reporter gene (G5EnXCAT) activity was decreased by GAL4-HIPK2 (Fig. 4C, construct B; 9-fold repression). Further deletion analysis revealed that HIPK2 contains a strong co-repressor domain (CRD, a.a. 97–230) at the N terminus (Fig. 4C, constructs F–M). These results strongly support the idea that HIPKs function as co-repressors for homeoproteins. Thus, as a co-repressor for homeoproteins, the interaction domain of HIPK2 is indispensable for interacting with homeoproteins (Fig. 4B). At the same time, the CRD is required for its co-repressor activity (Fig. 4C, construct K), presumably by serving as a protein-binding interface with a co-repressor complex or with the basal transcription machinery. Other domains such as the SRS and the YH domain showed a positive and a negative effect on co-repressor activity, respectively (Fig. 4C, constructs B–D and N–P). The GAL4-HIPK2(K221R) construct that contains a kinase inactive HIPK2-K221R fused to the GAL4 DNA-binding domain showed decreased repressor activity (Fig. 4C, construct Q; 40% reduction compared with that of the wild type GAL4-HIPK2), which is similar to the results obtained with the HIPK2-K221R mutant construct (Fig. 4B, lane 4). Because this effect is independent of interaction with homeoproteins, it is likely that for the full co-repressor activity of HIPK2, other unknown molecules such as components of a co-repressor complex or the basal transcriptional machinery may serve as substrate(s). HIPK1, which also localizes to nuclear speckles, showed co-repressor activity under the same experimental conditions.2

In summary, we have discovered a novel family of HIPKs that localize to nuclear speckles and provide the first evidence that HIPKs can act as transcriptional co-repressors for NK homeodomain transcription factors. Other co-repressors (N-CoR and SMRT) for a family of nuclear receptors were previously cloned and characterized (24, 25). However, HIPKs constitute a novel class of co-repressors in the sense that they have protein kinase activity. Because HIPKs also interact with HOX proteins (Table I), HIPKs provide important tools with which to study the mechanisms that control the functional specificity of homeoproteins in vivo and may help to decipher the HOX code (2).

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REFERENCES

1. McGinnis, W., and Krumlauf, R. (1992) Cell 68, 283–302
2. Krumlauf, R. (1994) Cell 78, 191–201
3. Mann, R. S., and Chan, S.-K. (1996) Trends Genet. 12, 258–262
4. Chan, S.-K., Jaffe, L., Capovilla, M., Botas, J., and Mann, R. S. (1994) Cell 78, 603–615
5. van Dijk, M. A., and Murre, C. (1994) Cell 78, 617–624
6. Pippel, H., Bienz, M., Studer, M., Chan, S.-K., Aparicio, S., Brenner, S., Mann, R. S., and Krumlauf, R. (1995) Cell 81, 1031–1042
7. Copeland, J. W. R., Nasiadka, A., Dietrich, B. H., and Krause, H. M. (1996) Nature 385, 162–165
8. Guichet, A., Copeland, J. W. R., Erdelyi, M., Hlousek, D., Zavorszky, P., Ho, J., Brown, S., Percival-Smith, A., Krause, H. M., and Ephrussi, A. (1997) Nature 385, 548–552
9. Yu, Y., Li, W., Su, K., Yussa, M., Han, W., Perrimon, N., and Pick, L. (1997) Nature 385, 552–555
10. Kim, Y., and Nirenberg, M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7716–7720
11. Bürghlin, T. R. (1994) in Guide Book to the Homeobox Genes (Duboule, D., ed.) pp. 27–71, Oxford University Press, Oxford
12. Harvey, R. P. (1996) Dev. Biol. 178, 203–216
13. Schott, J.-J., Benson, D. W., Basson, C. T., Pease, W., Silberbach, G. M., Moak, J. P., Maron, B. J., Seidman, C. E., and Seidman, J. G. (1998) Science 281, 108–111
14. Chen, C. T., Bartel, P. L., Steen glanz, R., and Fields, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9578–9582
15. Schubert, F. R., Fainsod, A., Gruenbaum, Y., and Gruss, P. (1995) Mech. Dev. 51, 99–114
16. Hwang, S. B., Kim, S. J., Nah, M. J., Lee, Y. M., Kim, Y., and Yoo, O. J. (1997) Gene (Amst.) 198, 373–378
17. Lee, Y. M., Park, T., Schulz, R. A., and Kim, Y. (1997) J. Biol. Chem. 272, 17531–17541
18. Hanks, S. K., and Hunter, T. (1995) in The Protein Kinase Facts Book (Hardie, G., and Hanks, S., eds.) pp. 7–47, Academic Press, San Diego, CA
19. Rogers, S., Wells, R., and Rechsteiner, M. (1986) Science 234, 364–368
20. Spector, D. L. (1993) Annu. Rev. Cell Biol. 9, 265–315
21. Hedley, M. L., Amrein, H., and Maniatis, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11254–11258
22. Krause, H. M., and Gehring, W. J. (1989) EMBO J. 8, 1197–1204
23. Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S., and Zoller, M. J. (1993) Trends Biochem. Sci. 18, 84–89
24. Horlein, A. J., Nia¨r, A. M., Heinzl, T., Torchia, J., Glos, B., Kurakawa, R., Ryan, A., Komet, Y., Siderstr¨om, M., Glaes, C. K., and Rosenfeld, M. G. (1995) Nature 377, 397–404
25. Chen, J. D., and Evans, R. M. (1995) Nature 377, 454–457