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The Metastasis Suppressor Candidate Nucleotide Diphosphate Kinase NM23 Specifically Interacts with Members of the ROR/RZR Nuclear Orphan Receptor Subfamily

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We have cloned proteins that interact with the nuclear orphan receptor RZRβ using the yeast two-hybrid system. We identified, amongst a number of other genes, the nucleoside diphosphate kinase (NDPK)-2 also known as Nm23-2, c-*myc* regulatory factor PuF and differentiation inhibitory factor. RZRβ specifically interacts with Nm23-2 but not with the closely related tumor metastasis suppressor candidate gene product Nm23-1. In contrast, RORα interacts with both Nm23 proteins. These findings were corroborated by *in vitro* interaction assays based on GST-pulldown experiments. With n-*myc* we propose a candidate gene regulated by RORα/RZRβ and Nm23, based on the finding that the respective DNA binding sites in the first intron are conserved in several mammalian species.

The receptors for steroids, vitamin D, thyroid hormone, and the retinoids are prominent members of the nuclear receptor superfamily [e.g., 1]. More members of this superfamily have been identified by homology cloning. They are known as orphan receptors, referring to the fact that their ligands are unknown [2,3]. The nuclear orphan receptors RORα, RZRβ and RORγ constitute a small subfamily identified independently by several groups [4-7]. RORα is expressed in a variety of organs with highest levels of specific mRNA detected in leukocytes [4]. Recently, it was found that the mouse mutation *staggerer* is caused by a disruption of the RORα gene [8]. Expression of the RZRβ gene appears to be restricted to the central nervous system. Its anatomical distribution suggests a role in sensory input integration and circadian biology [9,10]. RORγ is expressed at highest levels in muscle tissue [7] and might be involved in differentiation processes [A. Jetten, personal communication].

To learn more about the molecular mechanisms of ROR/RZR function, we performed a yeast two-hybrid screen with the aim to identify proteins that interact with these receptors. In this study we report the identification of Nm23 as a binding partner specific for RORα and RZRβ. We will discuss possible implications of our findings.

MATERIALS AND METHODS

Strains, plasmids, media, and microbiological techniques. The yeast strain Y190 (*MAT***a** *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112, GAL1-lacZ@URA3, GAL1-HIS3@LYS2 cyh*^{r}) was obtained from S. Elledge [11]. The mouse brain cDNA library in vector pGAD10 was from Clontech (Cat. No. ML4003AB, mouse strain BALB/c; Palo Alto, CA). Plasmid pRTH10 was constructed using pYTH9 as basic vector. A PCR-generated DNA fragment encoding the ligand-binding domain of rat RZRβ (Ser^{76} - end) was cloned between the *Eco*RI and *Bgl*II sites thus fusing it

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Abbreviations: AD, activation domain; BD, DNA-binding domain; GST, glutathione-S-transferase; LBD, ligand-binding domain; NDPK, nucleoside diphosphate kinase; Nm23, non-metastatic 23; PCR, polymerase chain reaction; ROR, retinoid acid receptor related orphan receptor; RT-PCR, reverse transcription PCR; RZR, retinoid Z receptor. In nucleotide sequences: D = A, G, or T; N = any nucleotide; R = A or G; W = A or T.

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downstream of the Gal4p DNA-binding domain (Gal4-BD). Plasmid pRTH15 was constructed in a similar way using a PCR-generated DNA fragment encoding the ligand-binding domain of human RORα (Ser^{79} - end) and pGBT9 as parent plasmid (Clontech, Palo Alto, CA). yeast and bacterial strains were propagated using standard methods. YPD and SD media have been described [12]. Yeast transformations were performed by the lithium acetate method as described [13]. For GST-pulldown experiments fusions between glutathione-S-transferase (GST) and the respective cofactor were constructed in the plasmid pGEX-4T1 (Pharmacia, Sweden).

Library screening. Plasmid pRTH10 was cut with *Xba*I and integrated at the TRP locus in the *Gal1-lacZ* and *Gal1-HIS3* reporter strain Y190 leading to Trp prototrophy. This strain was then transformed to Leu prototrophy with a library of plasmid pGAD10 containing mouse brain cDNAs fused to the Gal4 activation domain (AD; amino acids 768-881). Aliquots were taken from each transformation mix before plating and used to determine the transformation efficiency on medium lacking trp and leu. The transformation mix was then plated on 20×20 cm petri dishes containing SD medium lacking trp, leu, and his but including 30 mM 3-AT (Sigma), and incubated at 30°C for 3-5 days. His^+ colonies were then screened for β-galactosidase activity using a filter lift assay [14]. Colonies were transferred onto nylon filters (Hybond N, Amersham), permeabilized by freezing at −80°C (20 min.), and thawed at room temperature. Filters were then overlaid on Whatman 3MM paper saturated with an X-gal solution and incubated at 37°C overnight. Positive colonies were picked from the SD-trp,-leu,-his,+3-AT plates and grown overnight in 10 ml of YPD medium. DNA prepared from these cultures was transformed into *E. coli* to isolate and amplify the appropriate library plasmids. cDNA inserts were sequenced using flanking primers and cycle sequencing with fluorescent terminators (Applied Biosystems).

β-Galactosidase assays. β-galactosidase assays on plates were essentially carried out as described previously [14]. Briefly, cells were transferred from the selective media (SD-his-leu-trp+30mM 3AT) onto a nylon filter (Hybond N, Amersham), the filter was placed at −80 °C for approx. 20 min. and was then placed on a piece of filter paper soaked in a solution of 100 mg/l X-gal in 0.1 M $NaPO_4$ buffer pH 7.0 and incubated for the appropriate period of time. For β-galactosidase assays in liquid media, strains carrying the two-hybrid plasmids were grown to mid-log phase in selective SD-trp-leu medium. Cultures were diluted into YPD medium and incubated for 4-5 hr at 30°C before preparation and assayed for β-galactosidase as described previously [15,16]. Typically, four individual transformants of each plasmid combination were selected for liquid assay.

Interaction assay. GST-fusions were expressed in *E. coli* and purified on glutathione Sepharose 4B beads according to the manufacturer (Pharmacia, Sweden). The *in vitro* translation reactions were performed in the presence of [^{35}S]methionine (Amersham) using TNT extracts from Promega and the recombinant expression vector pSG5 encoding the human RZRα (the fourth N-terminal splicing variant of RORα) or rat RZRβ. Purified GST-fusion protein bound to glutathione Sepharose beads was incubated with aliquots of *in vitro*-translated RORα or RZRβ at room temperature for 30 min. Repeated washings with phosphate-buffered saline / 1% Triton X-100 at room temperature and incubation with 10 mM glutathione in 100 mM Tris-HCl (pH 8.0) released specifically bound protein which was detected by polyacrylamide gel electrophoresis and autoradiography of the dried gel.

RESULTS AND DISCUSSION

Isolation of Cofactors of RZRβ

To find proteins interacting with ROR/RZR, a two-hybrid screen was performed as described [17] using reagents as published [11]. Approximately 10^6 transformants were spread on SD-his, -leu, -trp, +30 mM 3-AT plates, selecting for the transcriptional activation of the *Gal1-HIS3* reporter construct. After selection, transformants were screened for their ability to produce β-galactosidase using a filter lift assay [14]. Since strain Y190 carrying the integrated RZRβ bait fusion alone generated some background β-galactosidase activity, only colonies displaying a positive signal after a maximum of 30 min. were isolated and further analyzed. A total of 60 colonies was subjected to further tests.

The library-derived plasmids from these colonies were extracted and recovered in *E. coli.* The inserts of the isolated plasmids were partially sequenced using primers derived from flanking sequences. The cDNAs encoding proteins strongly interacting with RZRβ fell into five classes: One class contained cDNAs encoding the mouse Nm23-2 (also known as nucleoside diphosphate kinase-2, NDPK-2), a second class contained the previously identified TRIP1 protein [18], and the cDNAs in the remaining three classes showed no obvious homology to any known sequence in the database.

Highly conserved homologues of Nm23 have been found in all eukaryotic organisms examined including prokaryotes, plants, insects and vertebrates where the gene is involved in normal

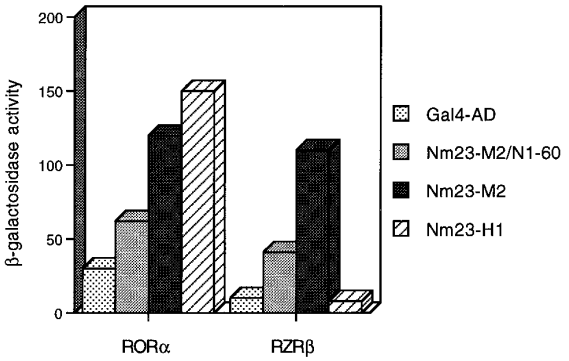

FIG. 1. Nm23 and RORα/RZRβ interact in the yeast two-hybrid system. β-Galactosidase assays reveal protein-protein interactions *in vivo* between Gal4BD-RORα or -RZRβ fusion products and Nm23-H1- or Nm23-M2-Gal4AD fusion products, respectively.

organogenesis and differentiation. There are two Nm23 genes, nm23-1 and nm23-2. They encode proteins of about 17 kD which are about 90% identical at the level of peptide sequence.

To characterize in more detail the interactions between Nm23 and ROR/RZR, several fusion constructs were created: the N-terminal 60 amino acids of mouse Nm23-2 predicted to be important for biological activity [19] were fused to Gal4p-AD; the human Nm23-1 was cloned by RT-PCR and was fused to Gal4p-AD; the RORα ligand-binding domain (LBD) was fused to the Gal4p DNA binding domain (BD; amino acids 1-147). The plasmids encoding the BD-ROR/RZR and AD-Nm23 fusions were introduced into yeast strain Y190 and tested for interaction using liquid β-galactosidase assays. Figure 1 shows that mouse Nm23-2 was interacting with RZRβ and even more strongly with RORα, while human Nm23-1 was specifically interacting only with RORα. Moreover, the N-terminal 60 amino acids of mouse Nm23-2 retaining the putative I-factor activity [19] were sufficient to generate a signal clearly above background with both RORα and RZRβ. We also tested other, related, nuclear receptors for their ability to interact with Nm23 (thyroid hormone receptor β, retinoic acid receptor β); however, there were no specific interactions with these nuclear receptors (data not shown). We propose, therefore, that the interaction between Nm23 and the ROR/RZR's is unique amongst the members of the nuclear receptor superfamily.

In Vitro Binding Assays

To show physical protein-protein interactions between the ROR/RZR's and the Nm23's we performed GST-pulldown experiments. Recombinant GST-Nm23's bound efficiently *in vitro*-translated [S^{35}]methionine-labeled RZR's (Fig. 2). The interactions were strongest between RORα and the GST-fusions of mouse Nm23-2 and human Nm23-1. RZRβ only weakly interacted with the human Nm23-1 while it bound much more efficiently to the murine Nm23-2. The N-terminal 60 amino acid portion of Nm23-2 conferred significant binding to both GST-ROR/RZR fusion proteins. The interactions were robust enough to persist in the presence of 1% Titron X-100. Taken together the *in vitro* binding assays essentially confirm the yeast two-hybrid transcription assays.

What Is the Function of the RZR/Nm23 Interaction?

The nm23 gene was originally identified in differential colony hybridization experiments performed on low and high metastatic murine melanoma cell sublines [20]. Nm23 RNA levels correlated inversely with metastatic potential [21]. Moreover, transfection of nm23 cDNA has

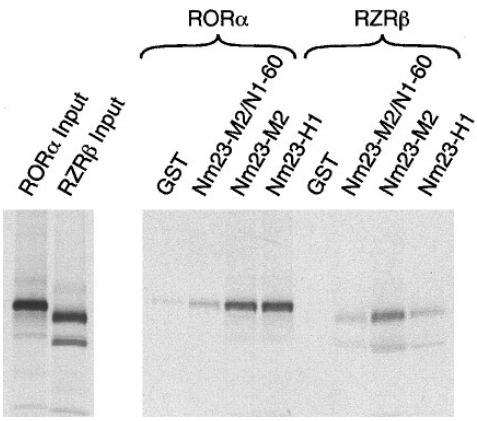

FIG. 2. Nm23 and ROR/RZR physically interact *in vitro.* The ability of various GST-Nm23 fusion proteins to interact with [S^{35}]methionine-labeled *in vitro*-translated RORα and RZRβ was evaluated in GST-pulldown assays.

been reported to suppress malignant progression in *Drosophila* and mammalian cells. Therefore, the nm23 gene has been proposed as a tumor metastasis suppressor gene. However, exceptions to this rule have been found in colonic neoplasms [22,23] and in advanced neuroblastoma tumors that are associated with N-*myc* oncogene amplification and metastasis [24].

The biochemical mechanism is unknown by which Nm23 and its homologues confer the modulation of metastatic processes and differentiation programs in various organisms and tissues. The product of the nm23 genes is an enzyme that catalyzes the transfer of a phosphate residue from NTP to NDP, hence its other designation: nucleoside diphosphate kinase (NDPK). It has been questioned whether this enzymatic activity is responsible for the various biological effects of nm23-1 and nm23-2 but no evidence was found supporting such a correlation [reviewed in 25]. Rather the phosphorylation state of a certain serine residue of Nm23-1 appears to correlate with the tumor metastasis suppression potential [26].

A number of additional functions has been proposed including a role for Nm23-2 as a cofactor modulating the transcriptional activity of certain promoters, like e.g., that of the c-*myc* gene [27]. Repeats of pyrimidine-rich DNA binding motifs (CCCACCC) have been reported as binding site of Nm23-2 in the promoter of the c-*myc* gene [28]. Moreover, it was shown that the NDPK activity is irrelevant for the DNA binding and *in vitro* transcriptional activity of Nm23-2. This parallels the finding that the differentiation inhibitory effect of Nm23-2 is not linked to the kinase activity either [19] as well as our finding that the first 60 amino acids are sufficient to encode significant binding activity to ROR/RZR. It is, therefore, tempting to speculate that the interaction between Nm23 and ROR/RZR is part of the mechanism underlying the biological activities of Nm23. It would be interesting to see whether point mutations of Nm23-H1 described in neuroblastomas [29] or the phosphorylation state of its Ser_{44} residue correlating with tumor metastasis suppression better than expression levels [26] affect the interaction with RORα.

We performed transient transfection experiments using a variety of cell lines to test whether Nm23-1 or Nm23-2 can augment or influence the transcriptional transactivation of either RORα or RZRβ. However, we could not see any effect, possibly because Nm23/NDPK is a ubiquitous factor present in saturating amounts with respect to ROR/RZR. In a computer-assisted search of the DNA sequence data base we identified in the first intron of the N-*myc* gene a putative response element for ROR/RZR as well as three putative binding sites for Nm23-2. Interestingly, these elements are conserved between human, mouse, and rat (Fig. 3). Again, in transient transfection assays using parts of this first intron sequence engineered

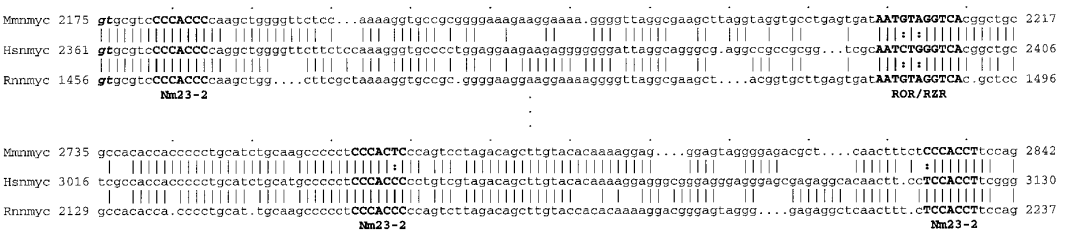

FIG. 3. Sequence comparison of part of the first intron of the mouse, human and rat N-*myc* genes. The numbers indicate the positions as deposited in the GenBank (Accession No. M12731, Y00664, X63281). The dinucleotide *gt* marks the 5′ splice site of the intron. The conserved putative binding sites for ROR/RZR (RORE: DAWNTRGGTCA) and Nm23-2 (CCCACCC) are highlighted in boldface letters.

upstream of the SV40 minimal promoter driving the expression of a reporter gene we were unable to see synergistic effects when both factors were over-expressed. We reason, therefore, that the cellular systems we used may hide the Nm23 effect on ROR/RZR because of high endogenous levels of the cofactor Nm23.

It is also conceivable, however, that ROR/RZR modulates the biological activity of Nm23 by binding to this enzyme and thereby influencing its DNA-binding behavior or other interactions it might have with cellular components leading to the biological phenomena observed. This would be reminiscent of the interaction of some disease-related proteins that contain stretches of polyglutamine (e.g., huntingtin) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; [30]) rendering GAPDH and its role in energy metabolism the cause of the observed disease phenomena.

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REFERENCES

1. Evans, R. M. (1988) *Science* **240,** 889–895.
2. O'Malley, B. W., and Conneely, O. M. (1992) *Mol. Endocrinol.* **6,** 1359–1361.
3. Laudet, V., and Adelmant, G. (1995) *Curr. Biol.* **5,** 124–127.
4. Becker-André, M., André, E., and DeLamarter, J. F. (1993) *Biochem. Biophys. Res. Commun.* **194,** 1371–1379.
5. Giguère, V., Tini, M., Flock, G., Ong, E., Evans, R. M., and Otulakowski, G. (1994) *Gen. Dev.* **8,** 538–553.
6. Carlberg, C., Hooft Van Huisduinen, R., Staple, J., DeLamarter, J. F., and Becker-André, M. (1994) *Mol. Endocrinol.* **8,** 757–770.
7. Hirose, T., Smith, R. J., and Jetten, A. M. (1994) *Biochem. Biophys. Res. Commun.* **205,** 1976–1983.
8. Hamilton, B. A., Frankel, W. N., Kerrebrock, A. W., Hawkins, T. L., FitzHugh, W., Kusumi, K., Russell, L. B., Mueller, K. L., van Berkel, V., Birren, B. W., Kruglyak, L., and Lander, E. S. (1996) *Nature* **379,** 736–739.
9. Schaeren-Wiemers, N., André, E., Schwab, M., and Becker-André, M. (1996) submitted.
10. André, E., Steinmayr, M., Brawand, C., and Becker-André, M. (1996) submitted.
11. Elledge, S. J. (1993) *Genes & Dev.* **7,** 555–569.
12. Rose, M. D., Winston, F., and Hieter, P. (1990) Methods in Yeast Genetics.
13. Schiestl, R. H., and Gietz, R. D. (1989) *Curr. Genet.* **16,** 339–346.
14. Breeden, L., and Nasmyth, K. (1993) *Cold Spring Harbor Symp. Quant. Biol.* **50,** 643–650.
15. Jarvis, E. E., Hagen, D. C., and Sprague Jr., G. F. (1988) *Mol. Cell. Biol.* **8,** 309–320. Miller, J. (1972) Experiments in Molecular Genetics.
16. Fields, S., and Song, O. (1989) *Nature* **340,** 245–246.
17. Durfee, T., Becherer, K., Chen, P-L., Yeh, S-H., Yang, Y., Kilburn, A. E., Lee, W-H., and Elledge, S. J. *Gen. Dev.* **7,** 555–569.
18. Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A., and Moore, D. D. (1995) *Nature* **374,** 91–94.

19. Okabe-Kado, J., Kasukabe, T., Hozumi, M., Honma, Y., Kimura, N., Baba, H., Urano, T., and Shiku, H. (1995) *FEBS* **363,** 311–315.
20. Steeg, P. S., Bevilacqua, G., Kopper, L., Thorgeirsson, U. P., Talmadge, J. E., Liotta, L. A., and Sobel, M. E. (1988) *J. Natl. Cancer Inst.* **80,** 200–204.
21. Bevilacqua, G., Sobel, M. E., Liotta, L. A., and Steeg, P. S. (1989) *Cancer Res.* **49,** 5185–5190.
22. Cohn, K. H., Wang, F., DeSoto-LaPaix, F., Patterson, L. G., Arnold, M. R., Weimar, J., Feldman, J. G., Levy, A. T., Leone, A., and Steeg, P. S. (1991) *Lancet* **338,** 722–724.
23. Haut, M., Steeg, P. S., Willson, J. K. V., and Markowitz, S. D. (1991) *J. Natl. Cancer Inst.* **83,** 712–716.
24. Keim, D., Hailat, N., Melhem, R., Zhu, X. X., Lascu, I., Veron, M., and Strahler, J. (1992) *J. Clin. Invest.* **89,** 919–924.
25. De La Rosa, A., Williams, R., and Steeg, P. S. (1995) *BioEssays* **17,** 53–62.
26. MacDonald, N. J., De La Rosa, A., Benedict, M., Perez-Freije, J. M., Krutsch, H., and Steeg, P. S. (1993) *J. Biol. Chem.* **269,** 25780–25789.
27. Postel, E. H., Berberich, S. J., Flint, S. J., and Ferrone, C. A. (1993) *Science* **261,** 478–480.
28. Berberich, S. J., and Postel, E. H. (1995) *Oncogene* **10,** 2343–2347.
29. Chang, C. L., Zhu, X-X., Thoraval, D. H., Ungar, D., Rawwas, J., Hora, N., Strahler, J. R., Hanash, S. M., and Radamy, E. (1994) *Nature* **370,** 335–336.
30. Burke, J. R., Enghild, J. J., Martin, M. E., Jou, Y-S., Myers, R. M., Roses, A., Vance, J. M., and Strittmatter, W. J. (1996) *Nature Medicine* **2,** 347–350.