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THE ORPHAN RECEPTOR TAK1 ACTS AS A REPRESSOR OF RAR-, RXR- and T3R-MEDIATED SIGNALING PATHWAYS

Takahisa Hirose[1,*], Rainer Apfel[2], Magnus Pfahl[2], and Anton M. Jetten[1,+]

[1]Cell Biology Section, Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health and Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

[2]San Diego Regional Cancer Center,11099 North Torrey Pines Road, La Jolla, California 92037

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Recently, we reported the cloning and characterization of the novel orphan receptor TAK1. In this study, we analyze the interaction of TAK1 with a variety of response elements (RE's) and demonstrate that TAK1 binds effectively to RE's composed of the core motif PuGGTCA configured in direct repeats spaced by one or more nucleotides. TAK1 bound poorly to palindromic or inverted palindromic motifs and was unable to bind to a single core motif, suggesting that a dimeric site is required for binding. Transfection experiments with CV-1 cells revealed that TAK1 is able to repress retinoid- and thyroid-hormone-induced transactivation through a subset of retinoid and thyroid hormone RE's. Our studies indicate that the antagonism of RAR-mediated transactivation does not involve the formation of heterodimers between TAK1 and RAR or RXR but is due to the competition of TAK1 homodimers with RAR-RXR heterodimers and RXR homodimers for binding to RARE and RXRE, respectively. Our results suggest that the orphan receptor TAK1 can be a negative modulator of the regulation of gene expression mediated by retinoid and thyroid hormone signaling pathways.

The nuclear receptor superfamily constitutes a class of ligand-responsive transcription factors that share a common modular structure consisting of five major domains (1-4). This superfamily includes receptors

* Current address: Department of Medicine III, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565,Japan.

+ To whom reprint requests should be addressed. Fax.: 919-541-4133; E-mail: jetten@niehs.nih.gov.

The abbreviations used are: RXR, retinoid X receptor; RAR, retinoic acid receptor; T3R, thyroid hormone receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; PPAR, peroxisome proliferator activating receptor; RE, response element; RA, retinoic acid; RARE, retinoic acid response element; CRBP, cellular retinol binding protein; RXRE, response element for RXR receptor; DR, direct repeat; P, palindrome.

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for steroid and thyroid hormones, retinoids and vitamin D, and a large number of structurally related proteins, termed orphan receptors, for which no ligand has yet been identified (1-4). The DNA-binding domain is the most conserved domain among the receptors and is composed of two "zinc-finger" motifs. This domain is involved in the interaction of the receptor with cognate response elements (RE's) in the promoter of specific target genes. Members of the largest subfamily of nuclear receptors which include retinoid, thyroid hormone and many orphan receptors, bind to direct or (inverted) palindromic repeats of the core motif PuGGTCA separated by spacers of defined length (1-4). Some nuclear receptors, such as RXR and the orphan receptor COUP-TF, can recognize their cognate RE's as homo-dimers. Others bind to RE's as a heterodimeric complex with another member of the nuclear receptor family. For example, the RAR, T3R, PPAR and vitamin D receptor, bind RE's as heterodimers with the RXR (1-4). The affinity of these complexes for their RE's is dependent on the configuration and spacing of the half-site motifs (1-4). Still other orphan receptors like ROR/RZR, NGF1-B/Nur77, and SF-1 bind as monomers to RE's composed of PuGGTCA preceeded by an AT-rich sequence (5-8). It has become increasingly evident that the different receptor networks interact with each other. For example, COUP-TF has been reported to be able to repress transactivation mediated by several nuclear receptors including retinoid, thyroid hormone and vitamin D receptors (9-11).

Previously, we described the molecular cloning and characterization of a novel orphan receptor, named TAK1 (also named TR4; 12), from human testis (13). In this study, we analyzed the interaction of this orphan receptor with a variety of RE's and demonstrate that TAK1 binds as a homodimer to several RE's and in particular to those composed of direct repeats of the sequence PuGGTCA spaced by one or more nucleotides. Through competition for the binding to these RE's, TAK1 interferes effectively with transcriptional activation mediated by retinoid and thyroid hormone receptors.

MATERIALS AND METHODS

Plasmids - The full length hTAK1 coding region T109 (13) was inserted into the *EcoR*I site of the plasmid pSG5. The pSG5 expression vectors containing the hRARα and mRXRα, respectively, were gifts from Dr. Chambon. The N-terminal truncated hRXRα expression vector was described previously (14).

Transfection assay - CV-1 cells (2 x 10^5 cells/well) were plated in 6-well dishes in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. The next day, transfections were carried out with Lipofectamine (BRL) following the method suggested by the manufacturer. The transfection cocktail (total volume of 200 µl) contained 2 µg of total plasmid DNA (expression vector, reporter gene vector and β-actin-LUC as internal control) and 6 µl of Lipofectamine in serum- and antibiotic-free MEM. Sixteen hrs after transfection retinoids or T3 were added, and 24 hours later, cells were harvested and lysates prepared. CAT assays were performed using ^{14}C-chloramphenicol (Amersham) as described (15,16); luciferase assays were performed using a luciferase assay kit from Promega according to the protocols provided by the manufacturer.

Site-directed mutagenesis - To construct hTAK1-117D in which Cys^{117} is converted to Asp, the Altered Site *in vitro* Mutagenesis System (Promega, Madison, WI) was used.

In vitro synthesis of TAK1 proteins and electrophoretic mobility shift assays (EMSA)- The TNT T7 RNA coupled reticulocyte lysate system (Promega) was

used to synthesize hTAK1, RARα and RXRα proteins. Probes for EMSA were radiolabeled by end-filling with Klenow polymerase (Stratagene). Approximately 0.5 ng of the probe was incubated with 5μl of programmed reticulocyte lysate and a buffer containing 10 nM Tris-HCl, 6% glycerol, 1 mM DTT, and 0.05% NP-40 in a final volume of 20 μl. 1 μg of poly(dI-dC) was included to prevent single-strand or non-specific binding. The reaction mixture was then loaded on a 6% nondenaturing polyacrylamide gel containing 0.25XTBE. As a control, probes were also incubated with the same amount of unprogrammed lysate. For antibody supershift analysis, 1 μl of a 1:10 dilution of the RARα monoclonal antibody (Affinity BioReagents, Neshanic Station, NJ) was preincubated with the reactions for 30 min at room temparature prior to the addition of the probe. Oligonucleotide sequences which are not provided in ref. 11 are: DR/h, gatc-TCAGGTCAgatc; IP0, gatccTGACCTAGGTCAgatcc; IP5, gatccTGACCTGA(C/G)T-CAGGTCAgatcc; IP9, gatccTGACCTGAGA(G/C)TCTCAG-GTCAgatcc.

Immunoprecipation assay -Immunoprecipitations were carried out as previously described (11). RXRβ protein and anti-RXRβ antibody were obtained from Affinity BioReagents.

RESULTS AND DISCUSSION

Binding specificity of TAK1 to natural and synthetic RE's- TAK1 belongs to a subfamily of nuclear receptors that contain a P-box composed of the sequence EGCKG. These receptors have been shown to bind to the consensus core motif PuGGTCA configured either as a direct repeat, palindrome, or inverted palindrome spaced by different numbers of nucleotides (3,4). In order to determine whether TAK1 can bind such motifs, the interaction of hTAK1 with a series of well-characterized natural and synthetic RE's (sequences are provided in ref. 11 and materials and methods) was examined by EMSA. As shown in Fig. 1A and B, hTAK1 was able to bind to several different natural and synthetic RE's. hTAK1 showed the highest level of binding for CRBPII-RXRE which is composed of four direct repeats of the core motifs spaced by one nucleotide. hTAK1 was also able to bind to the CRBPI-RARE, ApoA1-RARE and βRARE, present in the promoter of the cellular retinol binding protein I (CRBPI; 17), Apolipoprotein A1 (ApoA1; 18) and RARβ (19-21), respectively. hTAK1 had little affinity for the estrogen response element (ERE; P3), the rat malic enzyme (ME)-TRE (22), or the palindromic thyroid hormone response element (TREpal; P0)(23). Comparison of the binding of hTAK1 to several synthetic RE's indicated that it bound very efficiently to DR1, DR3, DR4 and DR5 composed of a direct repeat of the AGGTCA motif separated by one, three, four or five nucleotide(s), respectively (Fig. 2B). However, hTAK1 did not bind at all to the half-site motif (DR/h), suggesting that hTAK1 is unable to bind to the AGGTCA motif as a monomer. Moreover, hTAK1 did not bind the inverted palindrome IP0. These results suggest that TAK1 binds effectively to the RE's composed of direct repeats composed of the AGGTCA motif rather than to those in a palindromic or inverted palindromic

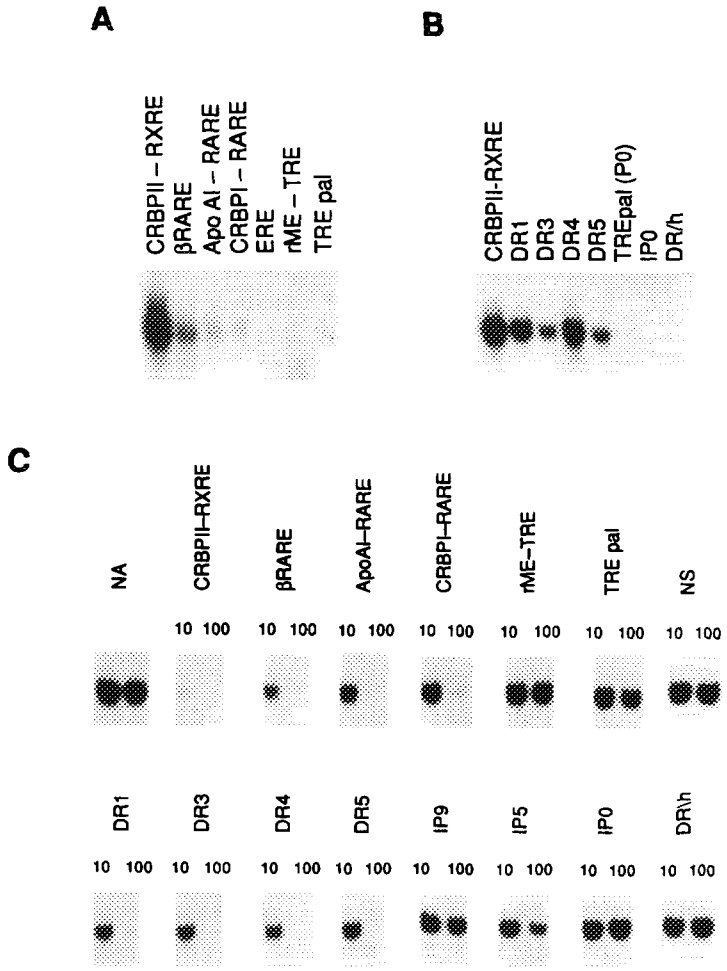

Fig. 1. Characterization of the binding specificity of hTAK1 to a variety of RE's. A.B. Analysis of hTAK1 binding to several natural (A) or synthetic (B) RE's. *In vitro* synthesized hTAK1 protein was incubated with ^{32}P-labeled RE's (equal amounts and specificity) for 30 min. The reaction mixtures were then analyzed by EMSA. C. Competition of various unlabeled RE's for the binding of TAK1 to DR1. *In vitro* synthesized TAK1 protein was incubated in the presence of a 10- or 100-fold excess of unlabeled RE's 30 minutes prior to the addition of ^{32}P-labeled DR1. The reaction mixtures were then analyzed by EMSA.

configuration. Whether the sequence flanking the core motif has any role to play in determining the affinity of TAK1 to an RE has yet to be determined.

The relative binding affinity of TAK1 was further tested by examining the ability of different RE's to compete with ^{32}P-DR1 for binding to hTAK (Fig. 1C). A 10-fold excess of unlabeled CRBPII-RXRE reduced the amount of ^{32}P-DR1 bound to hTAK1 dramatically while a 100-fold excess completely abolished DR1 binding. Although the synthetic direct repeats DR1, DR3, DR4 and DR5 and the natural RE's ApoA1-RARE, βRARE and CRBPI-RARE all were able to compete for binding, higher concentrations were required than for CRBPII-

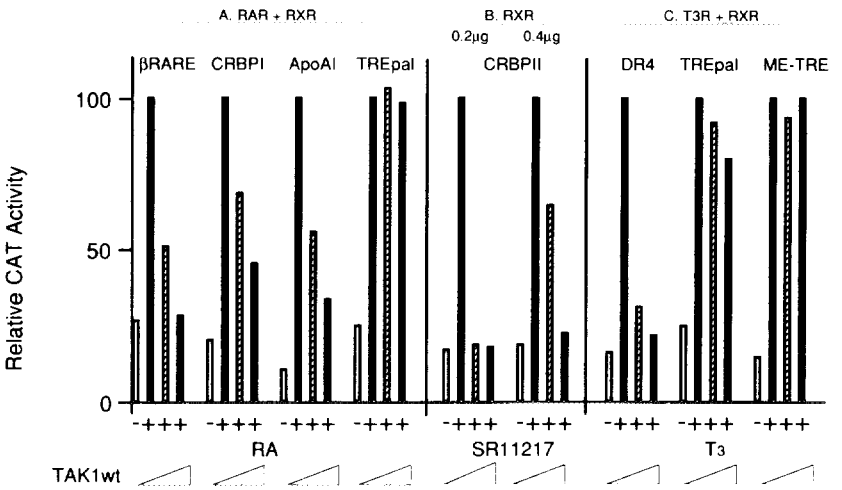

Fig. 2. Repressor function of hTAK1 on RAR, RXR, or T3R-mediated transactivation through a subset of RE's. RXRα and/or RARα or T3Rα expression vector (0.2 μg respectively except the right figure of 2B where 0.4μg of RXRα was used) was co-transfected with 0.2μg reporter plasmid into CV-1 cells with increasing amounts of wild type hTAK1 expression vector. The amount of DNA used in each transfection was equalized by the addition of empty expression vector. After transfection, cells were incubated in the presence or absence of 1μM all-*trans* RA, SR11217, or T3. Cells were harvested 24 hrs later and assayed for CAT activity. β-actin Luciferase vector was used as an internal control. The relative CAT activity was plotted as the percentage of the activity determined in drug-treated cells that were transfected with RAR/RXR/T3R and without TAK1. ☐ - indicates without drugs and TAK1 expression vector; ▩ - with drugs but without TAK1 expression vector; ▨ -with drugs and 0.4μg of TAK1 expression vector; ▩ -with drugs and 1.2μg of TAK1 expression vector. The results shown are representative for two independent experiments.

RXRE. The different synthetic DR's tested competed almost equally well for ^{32}P-DR1 binding while the palindromic or inverted palindromic RE's tested competed only weakly or were unable to compete.

TAK1 represses RAR, RXR, or T3R-mediated transactivation through a subset of RE's - The fact that hTAK1 was able to interact with RE's that have been reported to bind RAR-RXR or T3R-RXR heterodimers or the RXR homodimer suggested that it could influence the transactivation mediated by these receptors (24,25). To examine this possibility, the effect of TAK1 on the RARE-, RXRE- and TRE-dependent transactivation of a CAT reporter gene by RAR, RXR and T3R was examined in CV-1 cells (Fig. 2). In the absence of hTAK1, 10^{-6} M all-*trans* RA caused a 4- to 10-fold increase in the transactivation of the CAT gene from promotors with βRARE, CRBPI-RARE, ApoAI-RARE, or TREpal. Cotransfection with hTAK1 expression vector inhibited the RARE-dependent transactivation by RA in a dose-dependent manner but did not affect the RA-induced transactivation through TREpal. Likewise, TAK1 inhibited the RXRE-dependent transactivation by the RXR-specific retinoid SR11217 (10^{-6} M;26)(Fig. 2A,B). The effect of TAK1 on T3-induced transactivation was clear-

ly dependent on the TRE used (Fig. 2C). T3-induced transactivation through DR4 was inhibited by TAK1 but TAK1 did not affect TREpal- or rME-TRE-dependent transactivation. These results demonstrate that the effects of TAK1 on RARE-, RXRE- and TRE-dependent transactivation correlate well with the ability of TAK1 to bind to these RE's.

To examine the importance of the DNA-binding domain of hTAK1 in this inhibitory action, transfection experiments were carried out using a mutated TAK1 (TAK1-117D) in which Cys^{117} in the first "zinc finger" is replaced by aspartic acid. *In vitro*-translation of TAK1-117D was just as efficient as that of the wild type TAK1. In contrast to wild type hTAK1, cotransfection with the hTAK1-117D expression vector had no inhibitory effect on the RA-, SR11217-, or T3-induced transactivation of the reporter genes (Fig. 3). These results suggest that the inhibitory effect of TAK1 is not due to non-specific effects from overexpression of hTAK1 protein and indicate that the DNA binding domain of TAK1 is essential for this repression.

TAK1 binds to βRARE as a homodimer - To study the mechanism by which TAK1 represses the RARE-dependent transactivation by RAR-RXR heterodimers, we examined the possible role of an interaction of hTAK1 with RARα and RXRα in the binding to βRARE. In agreement with previous reports (see review in 3), RARα and RXRα bound to βRARE only as a heterodimer in EMSA (Fig. 4A, lanes 2-4). Inclusion of an anti-RARα antibody (Ab) caused a supershift of this complex (lane 11). Incubation of βRARE with RARα and a truncated form of RXRα yielded a complex that migrated slightly faster than the complex containing the wild type heterodimer (compare lanes 4 and 5).

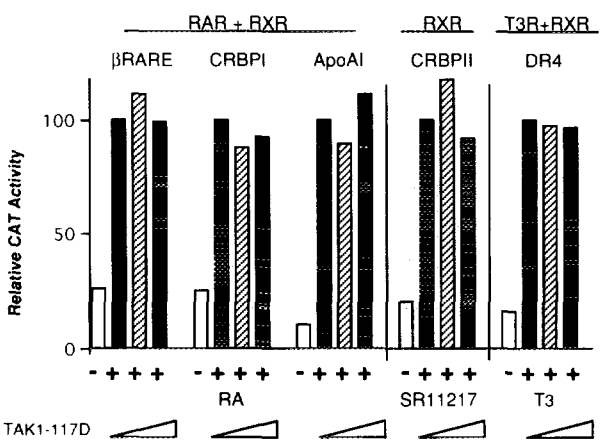

Fig. 3. Lack of repressor function of TAK1-117D on RAR, RXR, or T3R-mediated transactivation through a subset of RE's. Transfection experiments were performed as described in the legend of Fig. 2 except that TAK1-117D expression vector was used instead of the wild-type TAK1. Explanations for columns are the same as Fig. 2.

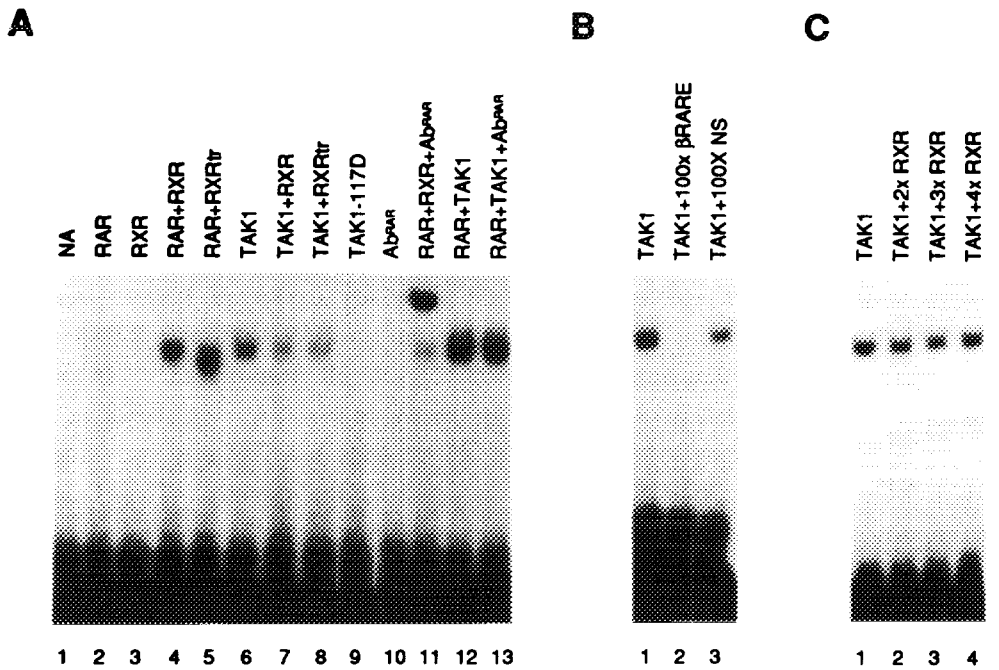

Fig. 4. Characterization of the binding of hTAK1 to βRARE. A. *In vitro*-synthesized RARα, RXRα, N-terminal truncated RXRα (RXRtr), hTAK1 and hTAK1-117D proteins were preincubated with ^{32}P-labeled βRARE at room temperature for 30 min in the combinations indicated. Some samples were incubated in the presence or absence of anti-RARα antibody (Ab). The reaction mixtures were then analysed by EMSA. Lane 1 represents binding of unprogrammed reticulocyte lysate. B. Competition by 100-fold excess unlabeled βRARE and a nonspecific oligonucleotide (NS) with ^{32}P-βRARE for hTAK1 binding. C. RXR does not influence binding of TAK1 to βRARE. *In vitro* translated TAK1 protein was incubated with ^{32}P-βRARE and increasing amounts of RXRα as indicated; samples were then analysed by EMSA.

hTAK1 formed a complex with βRARE that migrated at the same position as the RARα-RXRα/ βRARE complex, suggesting that hTAK1 binds to βRARE as a homodimer (lane 6). This binding is specific because addition of a 100-fold excess of unlabeled βRARE abolished the retarded band whereas the addition of a nonspecific oligonucleotide had little effect (Fig. 4B). In addition, the mutated receptor hTAK1-117D was unable to bind βRARE (lane 9) although the protein was correctly translated as assessed by *in vitro* translation (data not shown). Neither the presence of RXRα nor the truncated RXRα influenced the migration of the TAK1 complex (lanes 7 and 8). Moreover incubation of TAK1 with increasing amounts of RXRα does not affect binding of TAK1 to βRARE (Fig. 4C). The migration of the hTAK1-βRARE complex also did not change by the inclusion of RARα (lane 12). Moreover, addition of the RARα-monoclonal antibody did not cause a supershift (lane 13). These findings indicate that TAK1 does not bind to βRARE as a heterodimer with RARα or RXRα. Similarly, binding experiments with TAK1, RXR and CRBPII-RXRE

demonstrated that TAK1 bound to this RE as a homodimer and not as a TAK1/RXR heterodimer (data not shown). These observations support the conclusion that the repression of RARE- and RXRE-dependent transactivation by TAK1 is due to competition of TAK1 homodimers with RAR-RXR heterodimers and RXR homodimers, respectively, for binding to the RE.

The inability of TAK1 to form a heterodimer with RXR was further demonstrated by examining whether TAK1 is coimmunoprecipitated with RXR using antibodies raised against RXRβ protein. *In vitro* translated and ^{35}S-labeled RAR or TAK1 was incubated with bacterially expressed RXRβ protein. Each mixture was immunoprecipitated with an antibody against RXRβ and then analysed by denaturing polyacrylamide gel electrophoresis. *In vitro* translated and ^{35}S-labeled RAR or TAK1 are shown in Fig. 5A. As shown in Fig. 5B, RAR is coimmunoprecipitated with RXRβ antibody but TAK1 is not. Similar results were obtained using bacterially expressed RXRα protein. These results confirm that TAK1 is unable to form heterodimers with RXR.

In summary, our results show that TAK1 can act as a repressor of RAR-, RXR-, and T3R-mediated signaling pathways. At least in the case of TRE, this repressor activity of TAK1 is dependent on the TRE used. The inhibition of the RAR- and RXR-mediated transactivation appears to be due to competition of TAK1 homodimers with RAR/RXR heterodimers and RXR homodimers for binding to their respective RE's. We propose that the repression of the T3R-induced transactivation may be due to a similar mechanism and postulate that TAK1 may also inhibit Vitamin D receptor- and PPAR-mediated transactivation through DR3 and DR1 RE's, respectively. Identification of genes that are transcriptionally regulated by TAK1 as well as the endogenous RE's for TAK1 may indicate what role this repressor function plays in the regulation of differentiation, growth and development.

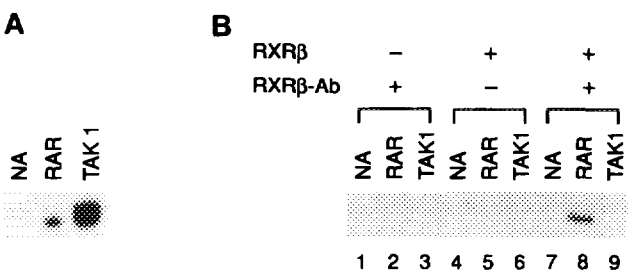

Fig. 5. TAK1 does not form a heterodimer with RXR. A. *In vitro* translated and ^{35}S-labeled RARγ and TAK1 protein were analysed by denaturing polyacrylamide gel electrophoresis. B. *In vitro* translated and ^{35}S-labeled RARγ or TAK1 were incubated in the presence (lanes 4-9) or absence (lanes 1-3) of bacterially expressed RXRβ. Proteins were immunoprecipitated with RXRβ-specific antibody (lanes 1-3 and 7-9) or with preimmune serum (lanes 4-6) using Protein G Sepharose. Radiolabeled, immunoprecipitated proteins were analysed by polyacrylamide gel electrophoresis and visualized by autoradiography. Unprogrammed reticulocyte lysate was used in lanes 1,4 and 7. The results shown are representative for two independent experiments.

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REFERENCES

1. Evans, R. M. (1988) Science 240, 889-895.
2 Green, S., & Chambon, P. (1988) Trends. Genet. 4, 309-314.
3. Giguere, V. (1994) Endocrine Reviews 15, 61-79.
4. Glass, C.K. (1994) Endocrine Reviews 15, 391-407.
5. Carlberg, C., van Huijsduijnen, R. H., Staple, J. K., Delamarter, J. F., & Becker-Andre, M. (1994) Mol. Endocrinol. 8, 757-770.
6. Giguere, V., Tini, M., Flock, G., Ong, E., Evans, R. M., & Otulakowski, G. (1994) Genes Dev. 8, 538-553.
7. Milbrandt, J. (1988) Neuron 1, 183-188.
8. Wilson, T. E., Fahrner, T. J., & Milbrandt, J. (1993) Mol. Cell. Biol. 13, 5794-5804.
9. Cooney, A. J., Leng, X., Tsai, S. Y., O'Malley, B. W., & Tsai, M-J. (1993) J. Biol. Chem. 268, 4152-4160.
10. Kliewer, S. A., Umesono, K., Heyman, R. H., Mangelsdorf, D. J., Dyck, J. A., & Evans, R. M. (1992) Proc. Natl. Acad. Sci. USA 89, 1448-1452.
11. Tran, P., Zhang, X-K., Salbert, G., Hermann, T., Lehman, J. M., & Pfahl. M. (1992) Mol. Cell. Biol. 12, 4666-4676.
12. Chang, C., Lopes Da Silva, S., Ideta, R., Lee, Y., Yeh, S., & Burbach, J. P. (1994) Proc. Natl. Acad. Sci. USA. 91, 6040-6044.
13. Hirose, T., Fujimoto, W., Yamaai, T., Kim K. H., Matsuura, H. & Jetten, A. M. (1994) Mol. Endocrinol. 8, 1667-1680.
14. Zhang, X-K., Hoffmann, B., Tran P., Graupner, G., & Pfahl, M. (1992) Nature 335, 441-446.
15. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular cloning; a laboratory manual. Cold spring harbor laboratory press.
16. Sato, B., Hirose, T., Kasayama, S., Takabayashi, H., Koga, M., & Nishizawa, Y. (1992) Intl. J. Oncol. 1, 415-423.
17. Smith, W. C., Nakshatri, H., Leroy, P., Rees, J., & Chambon, P. (1991) EMBO J. 10, 2223-2230.
18. Rottman, J. N., Widom, B., Nadal-Ginard, V., Mahdavi, V., & Karathanasis, S. K. (1991) Mol. Cell. Biol. 11, 3814-3820.
19. de The, H., Vivanco-Ruiz, M. M., Tiollais, P., Stunnenberg, H., & Dejean, A. (1990) Nature 343, 177-180.
20. Hoffmann, B., Lehmann, J. M., Zhang, X-K., Hermann, G., Graupner, G., & Pfahl, M. (1990) Mol. Endocrinol. 4, 1734-1743.
21. Sucov, H., Murakami, K. K., & Evans, R. M. (1990) Proc. Natl. Acad. Sci. USA 87, 5392-5396.
22. Desvergne, B., Petty, K. J., & Nikodem, V. M. (1991) J. Biol. Chem. 266, 1008-1013.
23. Umesono, K., Giguere, V., Glass, C. Rosenfeld, M. & Evans, R. M. (1988) Nature 336, 262-265.
24. Glass, C. K., Devary, O. V., and Rosenfeld, M. G. (1990) Cell 63, 729-738.
25. Mangelsdorf, D. J., Umesono, K., Kliewer, S. A., Borgelsdolf, D. J., Dyck, J. A., and Evans, R. M. 1991. (Cell) 66, 555-561.
26. Lehmann, J. M., Jong, L., Fanjul, A., Cameron, J. F., Lu, X. P., Haefner, P. Dawson, M. I., & Pfahl, M. (1992) Science 258, 1944-1946.