Nuclear factors specifically favor thyroid hormone binding to c-ErbAα1 protein (thyroid hormone receptor α) over-expressed in *E. coli*

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Abstract **A recombinant rat thyroid hormone receptor α (TRα or c-ErbAα1) was produced in *E. coli* as a non-mutated, non-fusioned protein and obtained as an efficient DNA and T3 binding protein that could be easily handled in a buffer-soluble state (rec-TRα). It was found that nuclear extracts (NE) added to rec-TRα markedly amplified not only DNA binding, which has been well documented, but also T3 binding (increased binding site concentration), which has not yet been reported. This T3 binding amplifying effect on rec-TRα occurs at low NE protein concentrations that produce no or minimal endogenous TR with respect to rec-TR, while similar concentrations of other proteins (e.g. ovalbumin or cytosol) only moderately enhanced T3 binding. The T3 binding amplifying nuclear factors, which are partly heat-labile, appeared as necessary auxiliaries in the analyses of partially purified rec-TRα. A protective effect of NE against a loss of affinity for T3 under the action of antibodies directed to certain sequences in the TRα D domain suggests that nuclear factors help rec-TRα to acquire and/or stabilize a conformation that allows the high affinity T3 binding. The nature of this nuclear amplifying factor is still unknown: RXRα which, produced in vitro, could amplify binding of the rec-TRα to a DNA thyroid response element, was unable to display such a rescue of high affinity binding sites.**

Key words: Thyroid hormone receptor α; Recombinant receptor; Hormone binding; Nuclear extract; Sequence-directed antibody; Retinoid X receptor α

1. Introduction

The thyroid hormone 3,5,3′-triiodo-L-thyronine (T3) activates nuclear T3 receptors (TR) which bind to thyroid response elements (TRE) in target genes. Two major TR isoforms have been identified, TRα and TRβ (β1 and β2) that are encoded by two homologous c-erbAα and β genes located on different chromosomes [1,2]. The TR belong to the steroid/thyroid/retinoid hormone nuclear receptor superfamily, a group of structurally related ligand-activated transcription factors. Like all nuclear receptors, TR possess a highly conserved cystein-rich DNA binding domain (C or DBD), and a long, less-conserved C-terminal ligand binding domain (E/F or LBD), both domains being separated by a short variable and flexible hinge domain (D). The LBD exhibits high specificity for the ligand and contains sub-regions involved in transcriptional activation/repression and in dimerization [3–5]. Recent findings indicate that the D domain must also be involved in hormone binding [6–9] and transcriptional activation [10]. A series of recent reports shows that TR interaction with TRE, and T3-mediated transcriptional activity of TR, are amplified by other nuclear auxiliary proteins (TRAP) [11,12], including the retinoid receptors RXR [13–15].

Analysis of functional regions in TR would be helped by the provision of sufficient amounts of purified receptors. Purification of TR from tissues was unsuccessful, due to low abundance and poor stability [16–18]. Later on, recombinant TR were produced from c-erbAα1 and β1 cDNAs mainly using *E. coli* [19–21]. Analysis and purification of recombinant TR are frequently hindered by their poor solubility which led to the use of detergents; this may impair T3 binding estimation and studies of structural changes that can occur after binding to hormone or DNA. We recently produced in *E. coli* an unfusioned, unmutated rat c-ErbAα1 protein (rec-TRα) that could be handled in a soluble form in low ionic strength buffer in the absence of detergents. We analyzed its reactivity towards several site-directed antibodies raised against different c-ErbAα peptides [9]. In this previous work, we observed that addition of serum proteins as well as ovalbumin, at 1 mg/ml, could induce an increase in the number of T3 binding sites detected in the rec-TRα preparations. Here we show for the first time that low amounts of nuclear extracts markedly and specifically enhance T3 binding to rec-TRα and become necessary auxiliary factors for estimation of T3 binding sites in purification protocols. These specific nuclear factors are most probably involved in acquisition and/or stabilization of a functioal conformation for T3 binding.

2. Materials and methods

2.1. Production and partial purification of recombinant rat thyroid hormone receptor α (rec-TRα)

The coding sequence of a rat c-erbAα1 cDNA (kindly given by R. Evans) was cloned under the control of a strong isopropyl-thiogalactoside (IPTG)-inducible promoter (pTrc99A vector; Pharmacia, France). Isolation of the synthetized protein was undertaken as previously described [9]. Briefly, 3 h after induction with 1 mM IPTG, cells were lysed in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) by successively adding lysozyme, Na-deoxycholate and DNase I. The cells were washed in the presence of 0.5% Triton X-100 and proteins extracted with 5 M guanidine-HCl in 20 mM Tris-HCl (pH 8.0). Protein refolding was provoked by a 10-fold dilution with buffer A (20 mM Tris-HCl, pH 8.0, 10% glycerol, 5 mM dithiothreitol (DTT)), containing 200 mM NaCl and, after 16 h at 0°C, by dialysing against four changes of buffer A plus 200 mM NaCl over 24 h. Rec-TRα was partially purified: the dialysed cleared bacterial extract was diluted to buffer A plus 150 mM NaCl and applied to an heparin-agarose column (Bio-Rad; France) previously equilibrated with buffer A plus 150 mM NaCl. After washing with the same buffer, the NaCl concentration was raised to 400 mM to elute TRα. An approximate 10-fold enrichment of TRα was then obtained leading to a specific activity of 700 pmol bound T3 per mg protein, when assayed as described below. T3 binding

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site recovery was approximately 45% of initial levels. Bacterial extracts and partially purified rec-TRα were supplemented with aprotinin (10 μg/ml) and stored as small aliquots at −80°C.

2.2. Preparation of nuclear extracts

Nuclei were purified from rat liver and from mouse Ob17 adipocytes as previously described [22,23]. Nuclei from rat hepatoma cells FAO which lack nuclear T3 binding sites [24] (kindly provided by Dr. Guillouzo, Rennes, France) were also purified as described for Ob17 cells [23]. Nuclear extracts were obtained using 400 mM KCl in 20 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, and generally stored as small aliquots at −20°C. Rat liver cytosol was prepared as the 100,000 × *g* supernatant of homogenate (1:4 w/v) in 0.32 M sucrose, 1 mM $MgCl_2$ and after an intermediate centrifugation step of 1000 × *g* to isolate the nuclei.

2.3. T3 binding assays

T3 receptor site concentration and affinity for T3 (K_a) were estimated in saturation experiments using [^{125}I]T3 (3 mCi/μg; Amersham, UK) in T3 binding buffer (20 mM Tris-HCl, pH 7.95, 1 mM $MgCl_2$, 100 mM KCl, 5 mM DTT) [22,23]. Incubations were performed at 0°C for 20 h. Non-specific binding was estimated in parallel assays in which was added an approx. 1000-fold excess of radioinert T3. Bound and free T3 were separated by the use of a Dowex 1X8 anion-exchange resin that binds free T3. Several comparative T3 binding assays were performed at 0.2 nM labelled T3 with or without 0.5 μM radioinert T3. These assays generally followed a preincubation (3 h, 0°C) of rec-TRα diluted in T3 binding buffer in the presence, or not, of the agents to be tested.

2.4. DNA binding assays

Binding of rec-TRα to DNA was studied, as previously described [9], by gel-electrophoresis mobility shift assay (EMSA) using a ^{32}P-labelled synthetic purified oligonucleotide (5′-CTAGTTCAGGTCATGACC-TGAA-3′) that after self-hybridization contains the palindromic T3 response element (TRE-pal): rec-TRα was preincubated for 2 h at 0°C with nuclear extracts (1 μg protein) or other agents to be tested, then for 30 min at 20°C with the ^{32}P-labelled TRE (30 fmol) in 50 μl DNA binding buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM $MgCl_2$, 50 mM NaCl, 2.5 mM DTT, 20% glycerol, 40 μg/ml poly d(I-C)). Protein–TRE complexes were separated from uncomplexed TRE by electrophoresis through 5% polyacrylamide gels in 0.5 × TBE and under 200 V at 4°C.

2.5. Production of rat RXRα by in vitro translation in reticulocyte lysate

The plasmid pRXR-T7, encoding rat RXRα, was kindly provided by Dr. J.A. Gustafsson [25]. RNA resulting from transcription under the T7 promoter was then translated in a reticulocyte lysate system in the presence or not of [^{35}S]methionine, according to the supplier's recommendations (NEN-Dupont, Les Ulis, France). Translation products were analyzed by SDS-PAGE (8–18%) and fluorography. A single ^{35}S-labelled band of the expected size was detected.

2.6. Other products

Antibodies to a c-ErbAα peptide sequence situated in domain D (amino acids 150–166) were obtained in rabbits and previously described as anti-α150 [8]. Retinoic acid (stereoisomer 9-*cis*) was obtained from Hoffman-Laroche (Basel, Switzerland). Protein concentrations were estimated according to Bradford's method, with serum albumin as the standard [26].

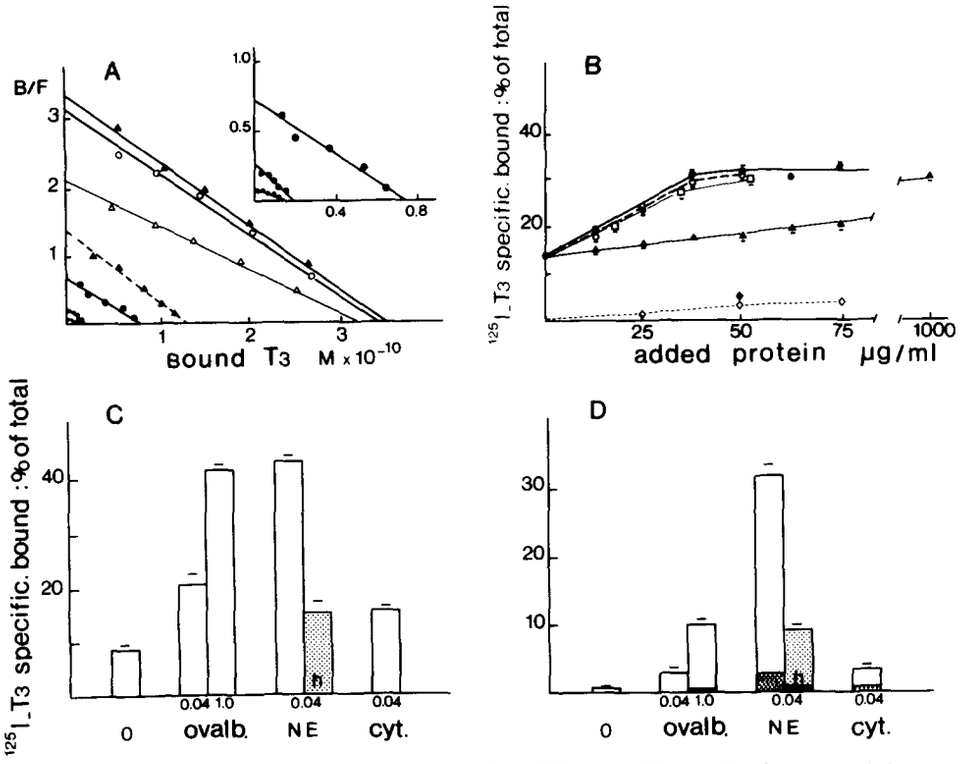

Fig. 1. Effect of nuclear extracts on T3 binding to recombinant c-ErbA α1 (rec-TRα). Rec-TRα in *E. coli* extracts (4–6 μg protein/ml: A, B, C), or partially purified (0.6 μg protein/ml: D), was preincubated (3 h, 0°C) in T3 binding buffer with or without the following agents: frozen nuclear extract (NE), ovalbumin (ovalb.), rat liver cytosol (cyt.) or serum, generally added at 0.04 or 1 mg protein/ml final concentration. Incubation with [^{125}I]T3 ± radioinert T3, and estimation of specific T3 binding were as described in section 2. (A) Scatchard plots of saturation analysis on rec-TRα with added: (●) buffer, (○) 0.04 mg/ml rat liver NE (RLNE) (▲—▲) and (▲- - -▲) ovalbumin at 1.0 or 0.04 mg/ml, respectively, (△) normal rabbit serum at 1 mg/ml; the lowest left regression lines, amplified in the insert, show T3 binding data to RLNE (0.04 mg/ml) alone before (●●) or after (••) freezing. (B) Effect of increasing concentrations of RLNE (●) or Ob17 adipocyte NE (○- - -○), FAO cell NE (□) and ovalbumin (▲) on [^{125}I]T3 (0.2 nM) binding to rec-TRα. Binding of [^{125}I]T3 to RLNE is shown by ◇ (in buffer) and ♦ (in the presence of 1 mg/ml ovalbumin). (C and D) Comparative effect of different proteins (0.04 or 1.0 mg/ml), and of heating RLNE (60°C, 10 min) (stippled bars), on [^{125}I]T3 (0.2 nM) binding to rec-TRα, in *E. coli* extract (C) or partially purified (D). T3 binding to ovalbumin, RLNE (before and after heating) and cytosol alone is shown by the shaded part of bars in D. Data values are the mean of duplicate assays in B (one representative of two experimental series) and mean ± S.E.M. of three different duplicate assays in C and D.

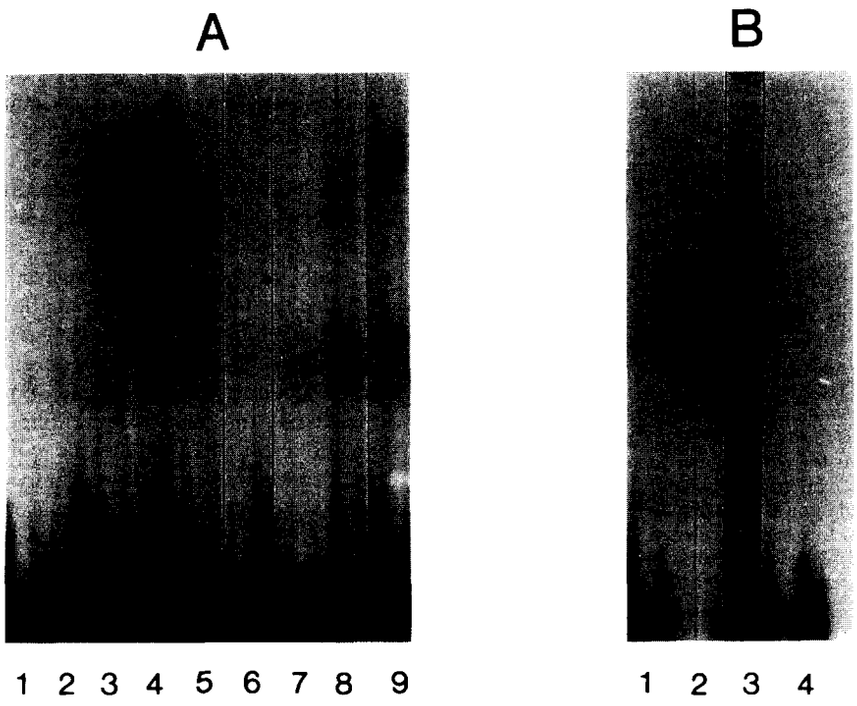

Fig. 2. Binding of recombinant rat c-ErbAα1 protein (rec-TRα) to DNA in electrophoresis mobility shift assays: effect of nuclear extracts (A) and of an in vitro produced rat RXRα (B). Rec-TRα in *E. coli* extract (2 μg protein) was incubated with a [^{32}P]TRE-pal (30 fmol) after a preincubation with rat liver (RL) or Ob17 adipocyte (ad) nuclear extracts (NE) (2.0 or 0.5 μg protein) or RXRα in rabbit reticulocyte lysate (RLys) (2 μl), in DNA binding buffer, and analyzed by EMSA as described in section 2. (A) Lanes: 1, control *E. coli* extract; 2 and 7, rec-TRα; 3, RLNE 2 μg; 4, rec-TRα+RLNE 2 μg; 5, rec-TRα+RLNE 0.5 μg; 6, rec-TRα+RLNE 2 μg+radioinert TRE-pal × 50; 8, adNE 2 μg; 9, rec-TRα+adNE 2 μg. (B) Lanes: 1, rec-TRα; 2, rRXRα in RLys (2 μl); 3, rec-TRα+rRXRα in RLys (2 μl); 4, same as 3+radioinert TRE-pal × 50.

3. Results and discussion

3.1. Nuclear factors markedly and specifically enhance T3 binding to recombinant thyroid hormone receptor α

We previously reported that T3 binding to rec-TRα in *E. coli* extracts was markedly enhanced when incubation with [^{125}I]T3 was performed in the presence of 1.0 mg/ml of either ovalbumin (which does not bind T3) or serum (which produced a very limited amount of T3 binding sites). This effect reflected an increase in the number of T3 binding sites. It was suggested that a high protein concentration could help diluted rec-TRα (4–8 μg/ml), stabilizing a proper conformation that would allow T3 to bind [9]. Fig. 1 shows that such an amplifying effect could be obtained, at least as efficiently, with low amounts of nuclear extracts (30–40 μg protein/ml). The effect was the same whether nuclear extracts were used after freeze–thawing (which provokes an important loss of detectable endogenous T3 binding sites) or freshly prepared (producing only a very low amount of T3 binding sites: approx. 500 fmol/mg protein [22], see Fig. 1A, insert). The T3 binding amplification by nuclear extracts also occurred without any significant change in the K_a for T3 (Fig. 1A). It was detected and completed within a low protein concentration range, a similar plateau being reached at approx. 40 μg protein/ml whether nuclear extracts were from rat liver (main TR content as β1 isoform), from mouse Ob17 adipocytes (TR content as α [8]), or from the TR-deficient FAO cells (Fig. 1B). At this concentration, ovalbumin (Fig. 1A–C) and rat liver cytosol (Fig. 1C) were far less efficient.

Moreover, and importantly, nuclear factors were found to play a major and unique role when T3 binding was studied with partially purified rec-TRα included in the binding tests at 0.4–0.8 μg/ml: without any protein addition a specific binding of T3 was barely detectable; ovalbumin, even at 1 mg/ml, or cytosol, poorly increased T3 binding; a marked amplification was obtained only with low concentrations of nuclear proteins (Fig. 1D). This amplification always led to a T3 binding site number which was far higher than the low level eventually produced by the nuclear extracts (shaded bars in Fig. 1D). In any case, heating the nuclear extracts (10 min, 60°C) partly reduced the amplifying effect (Fig. 1C and D). The applied heating conditions were known to be able to totally destroy, in nuclear extracts, any T3 binding activity [22] or any amplifying activity on TR binding to DNA [11]. Our present finding suggests that the nuclear factors that enhance T3 binding to rec-TRα may be heterogeneous. A cooperative effect of some nuclear auxiliary factors helping high affinity ligand binding was similarly and recently reported for the purified 1,25-dihydroxyvitamin D3 receptor [27] and the ecdysone receptor [28].

As evoked above, nuclear extracts are known to play an amplifying role in TR binding to DNA whether TR were produced by in vitro translation in reticulocyte lysate [11] or in bacteria [21]. Working with ^{32}P-labelled oligonucleotides that contained TRE sequences, different authors described both an increase in the amount of TR bound to TRE and qualitative changes with the formation of high Mr complexes with heat-labile nuclear TRAP (heterodimers and oligomers in EMSA) [12,13]. In the present study, using the EMSA methodology, a similar amplifying effect and similar changes in band pattern were observed when rec-TRα was incubated with rat liver nuclear extracts prior to [^{32}P] TRE-pal addition. This is illustrated

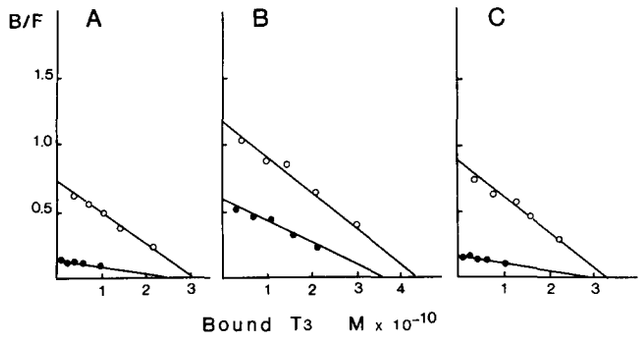

Fig. 3. Protective effect of nuclear extract (B) against an alteration of the T3 binding property of rec-TRα due to the action of anti-α150-166 antiserum compared to preimmune serum. Scatchard plots of saturation analysis with T3 performed on rec-TRα (5 μg/ml) sequentially preincubated (1) for 3 h at 0°C, with buffer in A, rat liver nuclear extract (35 μg/ml) in B, or ovalbumin (40 μg/ml) in C, (2) then for a further 3 h at 0°C after addition of preimmune (○) or anti-α150 (●) serum (1 mg protein/ml), before being incubated with increasing concentrations of [^{125}I]T3, as described in section 2. The final concentration of added proteins ranged between 1.00 and 1.04 mg/ml.

in Fig. 2 which also shows that nuclear extracts from Ob17 adipocytes give similar results with a slightly different mobility pattern in high Mr oligomeric bands.

The present results thus indicate that nuclear factors play an important and apparently specific role in stabilization or acquisition of two basal functions of rec-TRα: the effect on DNA binding is already well documented while the marked effect observed for hormone binding has not yet been reported.

We then considered two questions: (i) are nuclear factors specifically involved in acquisition/stabilization of a preferred conformation allowing high affinity T3 binding, or (ii) among different classes of nuclear factors that can be implied in this effect, are RXR, which are known to dimerize to TR, possible candidates to play this role?

3.2. *Nuclear factors are involved in the acquisition–stabilization of a prefered conformation that allows high affinity T3 binding*

To answer the first question we could make use of antibodies raised against several restricted sequences in the D and E/F domains of TRα and assay for their ability to inhibit T3 binding to TRα. Our previous results [8,9] indicate that both the extreme C-terminus and the C-terminal part of domain D play an important role in T3 binding to natural TRα and to an entire recombinant TRα as suggested by previous studies of T3 binding to deleted or mutated proteins [6,7]. More particularly, one set of our antibodies, directed against a c-ErbAα 150–166 sequence (anti-α150) displayed a different behaviour towards natural or recombinant TRα. It decreased the K_a for T3 binding to rec-TRα but not to natural unlinganded TRα prepared from Ob17 adipocytes, although immunorecognition was characterized in both cases [8,9]. Such a different reactivity to this set of antibodies evokes different structural states of the LBD surrounding the target sequence between both natural and recombinant TRα. An hypothesis was that natural TRα could be coupled to some nuclear proteins helping this TR acquire a conformation more resistant to interaction with anti-α150 antibodies.

To test this hypothesis, we attempted to reproduce in vitro a possible protective effect of nuclear factors: T3 binding was analyzed after sequential preincubations in the presence of (i) either buffer, nuclear extract or ovalbumin, and (ii) either antiserum to α150 peptide or preimmune serum (thus in the presence of 1.0–1.04 mg protein/ml, a concentration that per se increases the T3 binding site number; see open triangles in Fig. 1A). As shown in Fig. 3 and as cited above, anti-α150 dramatically decreased the K_a for T3 (panel A) when compared to preimmune serum (decrease of 74 ± 2% in 3 experiments). Interestingly, prior addition of rat liver nuclear extracts largely prevented rec-TRα from this dramatic decline of affinity for T3 due to the antibody (residual decrease of 38 ± 3% in 3 experiments) (panel B). This protective effect could not be reproduced when ovalbumin was used instead of nuclear extract (panel C). Nevertheless, this marked preventing effect of nuclear factors remained incomplete despite the use of an optimal nuclear extract concentration, and the affinity did not recover the level displayed with preimmune serum. This could be related to the in vitro conditions that did not allow a completed effect, or to the possible involvement of some post-translational modifications that cannot occur in this recombinant TR.

3.3. *The nuclear factor(s) that helps recombinant TRα acquire high affinity T3 binding is not a RXRα*

Many studies on TR binding to DNA have demonstrated that nuclear proteins, the so-called TRAP, markedly amplified the binding of rec-TRα to labeled TRE-pal (Fig. 2). Several authors reported that this TRAP family included the different RXR, particulary the widely distributed products of the RXR α and β genes [15,29]. These RXR proteins have been shown to increase the binding of TR to TRE by forming heterodimers, as demonstrated by the presence of DNA–protein complexes that displayed a lower mobility in EMSA. Moreover, RXR may also amplify the transcriptional responses to T3-activated TR, as suggested by results of co-transfection experiments [13–15,30,31]. The addition of 9-*cis* retinoic acid, a specific ligand of RXR, may or may not synergistically increase dimerization with, and transactivation by, TR [30,31]. A decreased heterodimerization of TR to RXR under 9-*cis* retinoic acid action that favored RXR homodimerization has also been reported [32].

In order to see whether RXR could be involved in the amplifying effect of nuclear extracts on T3 binding, and since what

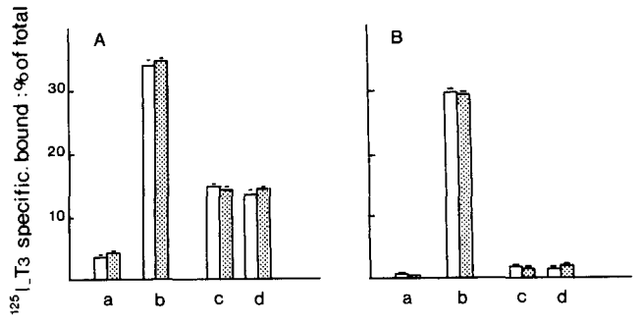

Fig. 4. Analysis of the effect of RXRα and its ligand 9-*cis* retinoic acid on the T3 binding property of recombinant TRα. Rec-TRα in *E. coli* extract (6 mg/ml) (A) or partially purified (0.5 mg/ml) (B) were preincubated for 3 h at 0°C in the presence of either buffer (a), rat liver nuclear extract (40 mg/ml) (b), rabbit reticulocyte lysate RXRα (c) or control (d) (50 mg/ml) without (open bars) or with (stippled bars) added 100 nM 9-*cis* retinoic acid, then for 18 h at 0°C with 0.2 nM [^{125}I]T3. Data are the mean of duplicate assays in one out of three similar experiments.

we have described could reflect previous reports on RXR-TR interactions, we first sought a possible interference with 9-*cis* retinoic acid. Fig. 4 shows that 100 nM of this RXR ligand was without any significant effect on T3 binding to rec-TRα in the absence or presence of nuclear extracts.

We then synthetized a rat RXRα, described as abundantly expressed in rat liver [29], by in vitro transcription/translation from a full-length cDNA. This RXRα preincubated with rec-TRα allowed an amplification of binding of the receptor to [^{32}P] TRE-pal with the formation of heterodimers, as shown in Fig. 2B, and as expected. Nevertheless, RXRα did not increase T3 binding to rec-TRα when comparing assays with controls that contained the same amount of unprogrammed lysate, whether 9-*cis* retinoic acid was present or not, and whether working with rec-TRα in *E. coli* extracts (Fig. 4A) or partially purified (Fig. 4B). RXR gene products, at least of the α-type, therefore seemed not to be the T3 binding amplifying factors. The possible involvement of other RXR or other nuclear factors/proteins (including other nuclear receptors) has to be explored, as well as the possible involvement of chaperone proteins, although it has been reported that TR is translated in the DNA binding state and not associated with hsp90 [33].

In conclusion, our results reveal for the first time that nuclear factor(s) are specifically implied in promoting the structural maturation of a recombinant TRα that leads to acquisition of the high affinity T3 binding site. With this specific role, nuclear extracts are thus important auxiliaries in studies of recombinant TR during purification protocols. In the present work this property is observed in vitro on recombinant TRα which was denaturated and renaturated. The same results were obtained when using recombinant TR directly extracted from bacteria by sonication without any denaturing agents, a procedure that gave a lower TR extraction yield [9]. These results are reminiscent of a similar role played by a nuclear extract sub-fraction that was demonstrated during purification of natural TR. In a previous study, we demonstrated that this sub-fraction had to be added to partially purified TR in order to restore high affinity T3 binding sites [34]. It is thus conceivable that in the native state in nuclei, TR, at least of the α type, exist in a more-or-less loose association with factor(s) which would help to complete and stabilize the quaternary structure of the TR LBD. These factor(s) do not seem to imply the widely represented RXRα, and thus differ, at least in part, from those already described for efficient target DNA binding. Analysis of their nature deserves further investigation to understand TR function and facilitate structural studies on the recombinant over-expressed receptor.

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