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RXRα, a promiscuous partner of retinoic acid and thyroid hormone receptors

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Retinoic acid receptor (RAR), thyroid hormone receptor (T_3R) and vitamin D_3 receptor (VD_3R) differ from steroid hormone receptors in that they bind and transactivate through responsive elements organized as direct rather than inverted repeats. We now show that recombinant RAR and T_3R are monomers in solution and cannot form stable homodimeric complexes on their responsive elements. Stable binding of the receptors to their responsive elements requires heterodimerization with a nuclear factor. This auxiliary factor is tightly associated with RAR and T_3R in the absence of DNA and co-purifies with both receptors. As demonstrated by extensive purification, the same auxiliary factor is required for stable DNA binding of RAR as for that of T_3R; the factor also facilitates the formation of a stable VD_3R–DNA complex. The auxiliary factor is identical to the retinoid X receptor α (RXRα) by biochemical and functional criteria. The identification of RXRα as a dimerization partner for the RARs, T_3Rs and VD_3R has important implications as to the function of these receptors and their ligands in development, homeostasis and neoplasia.

Key words: heterodimerization/retinoic acid receptor/retinoid X receptor/thyroid hormone receptor

Introduction

Retinoic acid (RA), thyroid hormone (T_3) and vitamin D_3 (VD_3) have multiple functions in the development and homeostasis of the vertebrate organism. The receptors for these hormones are closely related to each other and, more distantly, to the steroid hormone receptors, together forming the steroid/thyroid hormone superfamily of ligand-inducible transcription factors. Three receptor subtypes for RA (RARα, β and γ), two for T_3 ($T_3Rα$ and β) and a single VD_3 receptor (VD_3R) have been identified (Petkovich *et al.*, 1987; Giguere *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988; Krust *et al.*, 1989; Zelent *et al.*, 1989; Sap *et al.*, 1986; Baker *et al.*, 1988).

In the current model of steroid hormone action, the hormone binds to the monomeric receptor and induces nuclear translocation and/or dimerization of the receptor. The homodimeric, ligand activated receptor subsequently binds its cognate responsive element and activates transcription (Tsai *et al.*, 1988; Kumar and Chambon, 1988). The binding of the receptor to DNA as a homodimer dictates the structure of the hormone responsive element consisting of palindromically arranged half-sites (Luisi *et al.*, 1991).

The DNA binding domain of the receptors is highly conserved and contains eight cysteines tetrahedrically coordinating zinc to form two zinc fingers (Luisi *et al.*, 1991 and references therein). By mutational analyses, three amino acids at the base of the first zinc finger (the P-box) have been shown to be critical for the discrimination between responsive element half-site sequences (Umesono and Evans, 1989; Mader *et al.*, 1989; Danielson *et al.*, 1989). The P-box amino acids are located in a short α-helical structure that makes direct contacts in the major groove with the nucleotides in the responsive element half-sites (Luisi *et al.*, 1991). A more distal element (the D-box) has been shown to be important for determining the spacing between such half-sites (Umesono and Evans, 1989) forming a DNA-dependent dimerization interface (Luisi *et al.*, 1991).

The C-terminal part of the steroid hormone receptors contains the ligand binding and the major DNA-independent dimerization functions. The dimerization interface located in the E region encompasses several short hydrophobic heptad regions compatible with the formation of a coiled coil interaction between the receptor monomers (Fawell *et al.*, 1990). Similar conserved heptad repeat regions are present in the T_3R/RAR subgroup of nuclear receptors (Forman and Samuels, 1990).

Two recent findings suggest that the T_3R/RAR receptor subgroup differs functionally from the conventional model of the steroid hormone receptors. Firstly, these receptors bind their responsive elements in the absence of ligand (de Thé *et al.*, 1990; Damm *et al.*, 1989; Sap *et al.*, 1989; Sone *et al.*, 1990). Secondly, the recent characterization of natural RA, T_3 and VD_3 responsive elements has revealed that they consist of half-sites organized as direct repeats (Vivanco Ruiz *et al.*, 1991; Näär *et al.*, 1991; Umesono *et al.*, 1991). The binding of the homodimeric receptor to a direct repeat element would probably exclude protein–protein interactions between the D-boxes.

Several groups have reported the existence of nuclear proteins or activities which enhance the binding of T_3Rs, RARs and VD_3R to their responsive elements (Murray and Towle, 1989; Burnside *et al.*, 1990; Glass *et al.*, 1990; Sone *et al.*, 1990; Beebe *et al.*, 1991; Darling *et al.*, 1991; MacDonald *et al.*, 1991; O'Donnell *et al.*, 1991). Furthermore, Glass and coworkers reported that RAR and T_3R form heterodimers in *in vitro* binding cooperatively to some T_3 responsive elements (Glass *et al.*, 1989).

Using the vaccinia virus system we have assessed the protein–protein and protein–DNA interactions of the T_3R/RAR group of receptors. We found that both T_3R and RAR are closely associated with a nuclear protein. Heterodimerization of RARs, T_3Rs and possibly VD_3R

with the same protein is required for stable interaction of the receptors with their responsive elements. Surprisingly, the protein is functionally and biochemically indistinguishable from the retinoid X receptor α (RXRα), a protein previously proposed as a novel receptor for retinoids (Mangelsdorf *et al.*, 1991).

Results

T_3R and RAR DNA binding complexes

The chicken $T_3R\alpha$ and human RAR (α, β and γ) were expressed in HeLa cells using the vaccinia virus system as described previously (Vivanco Ruiz *et al.*, 1991). Receptor complexes present in extracts of HeLa cells infected with the respective viruses were revealed by mobility shift assays using the natural T_3RE located in the LTR of Moloney murine leukemia virus, MoMLV-T_3RE, (Sap *et al.*, 1990) and the RARE-β_2 element found in the promoter of the human and mouse RARβ gene (de Thé *et al.*, 1990; Vivanco Ruiz *et al.*, 1991; Sucov *et al.*, 1990). The T_3RE-MoMLV consists of a direct repeat of the sequence GGTC$^{A}/_{C}$ spaced by five nucleotides and mediates a T_3 but not an RA response. Conversely, the RARE-β_2 element consists of a direct repeat of the sequence GTTCA spaced by six nucleotides and mediates an RA but not a T_3 response (Vivanco Ruiz *et al.*, 1991; Umesono *et al.*, 1991; Näär *et al.*, 1991). As a comparison, the artificial responsive element, palindromic T_3RE (T_3RE-pal) was used. This element confers T_3 and RA responsiveness to a heterologous promoter (Umesono *et al.*, 1988).

In extracts containing $T_3R\alpha$, three different complexes (I, II and III) can be detected using oligonucleotides comprising the T_3RE-pal and T_3RE-MoMLV elements (Figure 1A, lanes 1–6). Complex I can also be detected with the RARE-β2 (Figure 1A, lane 7 and Vivanco Ruiz *et al.*, 1991). Similar results were obtained with $T_3R\beta$ and when extracts were prepared from other cell lines such as COS and RK13 (Vivanco Ruiz *et al.*, 1991 and data not shown). All three complexes contain T_3R protein as shown by supershift assays using $T_3R\alpha$ specific antibodies (Figure 1B, lanes 5–10). Complex III appears to consist of one T_3R monomer bound to the DNA as this is the only complex detected with oligonucleotides containing one T_3RE half-site sequence (data not shown). To assess the composition of complexes I and II, HeLa cells were infected with two viruses expressing full-length (p46-$T_3R\alpha$) and an N-terminally truncated $T_3R\alpha$ (p40-$T_3R\alpha$) using different virus titre ratios, thus expressing the two receptors at different relative concentrations in the infected cells. A novel type II complex was observed with these extracts (arrow in Figure 1C). A similar novel type II position was obtained with the MoMLV-T_3RE (data not shown). The occurrence of this complex is accompanied by a simultaneous and gradual disappearance of the p40 and p46 type II complexes (Figure 1C). We therefore conclude that this complex contains two receptor molecules bound to the responsive element; binding does not appear to be strongly cooperative as determined by bandshift experiments using purified T_3R monomer protein (data not shown). We have been unable to detect an intermediate type III complex which is in agreement with our assessment that this complex contains only one T_3R monomer. Similarly, we have been unable to detect an intermediate type I complex in these extracts even in high resolution gels or when using extracts from cells co-infected with p46-T3Rα and HIV gag extended $T_3R\alpha$ (data not shown). Therefore, we tentatively conclude that complex I consists of one T_3R monomer and an endogenous nuclear protein, referred to as 'X'.

A single complex was detected with extracts containing RAR (Figure 1A, lanes 2, 5 and 8) irrespective of the relative orientation and spacing of the half-site sequence elements. The formation of this complex is sequence specific (Vivanco Ruiz *et al.*, 1991) and contains at least one RAR monomer as shown by supershift experiments (Figure 1B, lanes 2–4 and 11–13). To assess whether this complex consists of an RAR homodimer, extracts were made which contain truncated RARβ lacking the A-region (ΔA-RARβ) and RARβ fused to HIV *gag* sequences (p24-RARβ) in different relative receptor concentrations (Figure 1E, lanes 1–6). The p24-RARβ–DNA complex appears as several bands in high resolution gels due to the HIV *gag* moiety (lane 6). Surprisingly, an intermediate type complex diagnostic for the presence of a receptor homodimer could not be detected. Furthermore, we were unable to detect dimerization between the different RAR subtypes using different receptor truncations and N-terminal fusions (data not shown). We tentatively conclude that the RAR forms a complex with an endogenous nuclear protein ('X') and that this complex binds to oligonucleotides comprising the RARE-β_2 and the T_3RE-pal.

RAR and T_3R heterodimer complexes

It has been reported that RAR and T_3R can heterodimerize (Glass *et al.*, 1989). We therefore investigated whether the auxiliary nuclear factor(s) as defined above could be endogenous RAR or T_3R. For this purpose, cells were infected with RARα and $T_3R\alpha$ expressing viruses and complex formation was studied in bandshift assays using the MoMLV-T_3RE and the T_3RE-pal (Figure 1D). A new complex (indicated by an arrow) was detected and the appearance of this complex was accompanied by a gradual disappearance of the T_3R type II and III and RAR complexes. Supershift experiments using RAR and T_3R specific antibodies demonstrated that this novel complex contains RAR and T_3R protein (data not shown).

The heterodimer complex appears to migrate at the same position as the T_3R type I complex (Figure 1D compare lanes 8 with lanes 9–11). The T_3R type I complex is barely visible using the T_3RE-pal but is much more abundant using the MoMLV-T_3RE probe (Figure 1D, compare lanes 2 and 8). The more prominent RAR–T_3R complex formed with the MoMLV-T_3RE is probably due to cooperative DNA binding. Because the T_3R type I complex migrates in the gel at the same position as the T_3R–RAR complex, it is not possible to determine unequivocally whether or not the T_3R type I complex consists of an RAR–T_3R heterodimer formed with endogenous RAR present in HeLa cells. However, antibodies specific for the three RAR subtypes did not affect the mobility of the T_3R type I complex (Figure 1B lane 9 and data not shown). To assess further the protein composition of the T_3R type I complex, we infected cells with ΔA-RARβ and $T_3R\alpha$ expressing viruses and bandshift assays were performed using the MoMLV-T_3RE probe. The recombinant ΔA-RARβ is in vast excess over the presumed endogenous HeLa RAR in these extracts.

If the T_3R type I complex contains one T_3R and one RAR moiety, we would expect that a novel complex would be obtained migrating in the gel with a slightly faster mobility than the presumed endogenous RAR−recombinant $T_3R\alpha$ complex because of the RARβ N-terminal truncation. Furthermore, we would expect that the T_3R type I complex would gradually disappear upon co-expression of increasing levels of ΔA-RARβ. As shown in Figure 1E (lanes 8−12), we do observe a novel ΔA-RARβ−$T_3R\alpha$ complex with the anticipated mobility, but neither the formation nor the

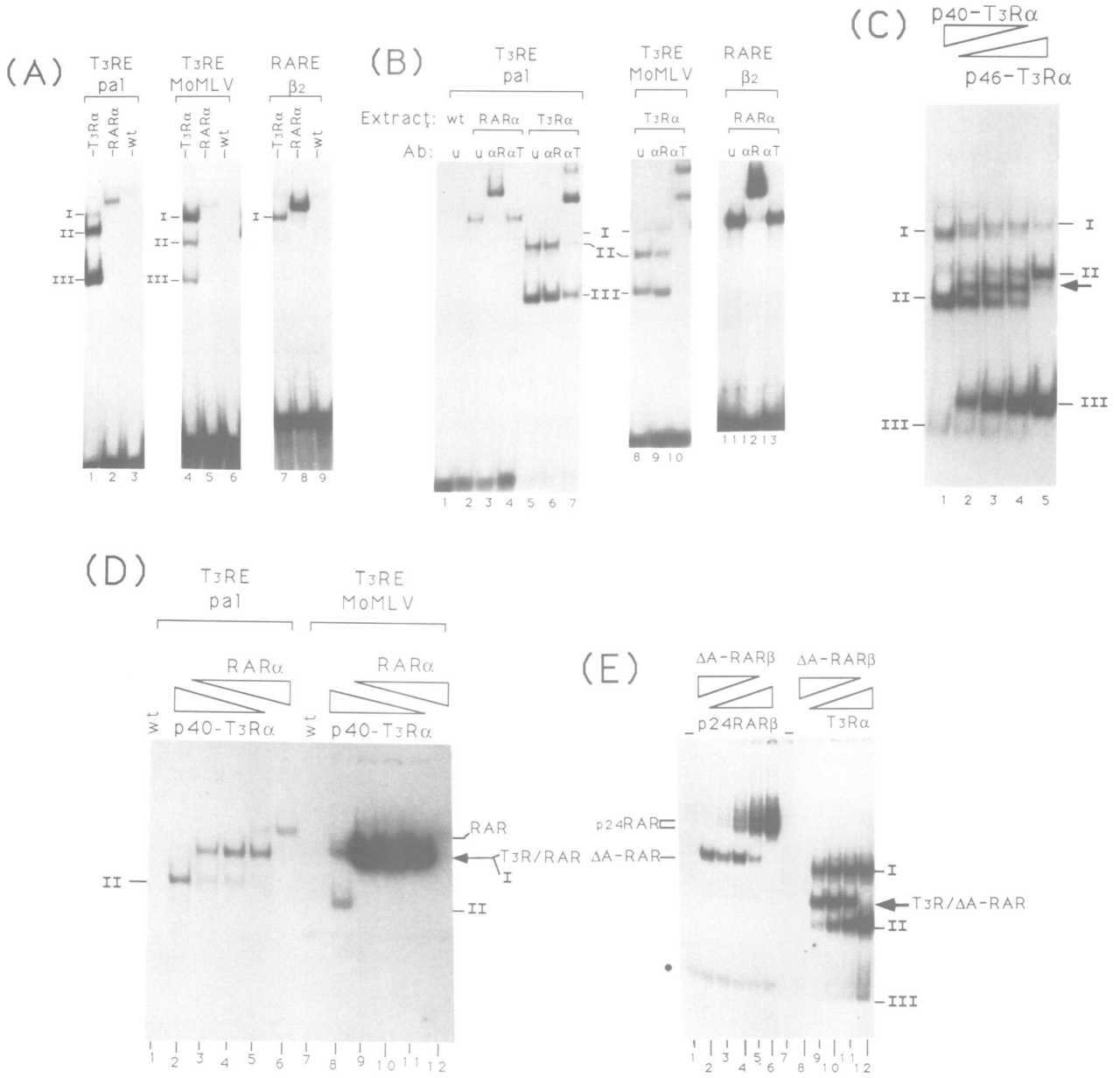

Fig. 1. Detection of protein−DNA complexes of RARs and T_3Rs with their responsive elements in gel retardation assays. (**A**) Complexes formed on T_3RE-pal oligonucleotide (lanes 1−3), T_3RE-MoMLV (lanes 4−6) and RARE-β2 (lanes 7−9) with $T_3R\alpha$ extracts (lanes 1, 4 and 7), RARα extracts (lanes 2, 5 and 8) or control extracts from wild type vaccinia virus infected cells (lanes 3, 6 and 9). (**B**) Extracts containing RARα (lanes 2−4 and 11−13), $T_3R\alpha$ (lanes 5−10) or control extract (lane 1) were mixed with T_3RE-pal (lanes 1−7), T_3RE-MoMLV (lanes 8−10) and RARE-β2 (lanes 11−13) and protein−DNA complexes were allowed to form. $T_3R\alpha$ antibodies (lanes 4, 7, 10 and 13), RARα antibodies (lanes 3, 6, 9 and 12) or unrelated antibodies (lanes 1, 2, 5, 8 and 11) were added to the mixture prior to separation of the protein−DNA complexes. (**C**) Protein−DNA complexes formed with T_3RE-pal and extracts from cells co-infected with p40-$T_3R\alpha$ and p46-$T_3R\alpha$ expressing viruses in variable virus titre ratios. The arrow indicates the novel type II complex. (**D**) Protein−DNA complexes formed between T_3RE-pal (lanes 1−6), T_3RE-MoMLV (lanes 7−12) and p40-$T_3R\alpha$ extracts (lanes 2 and 8), RARα extracts (lanes 6 and 12) and cells coinfected with different ratios of p40-$T_3R\alpha$ and RARα expressing virus (lanes 3−5 and 9−11) and wild-type virus control extracts (lanes 1 and 7). RAR complex and T_3R complexes I and II are indicated. The arrow indicates the RAR−T_3R heterodimer complex. (**E**) Protein−DNA complexes with RARE-β2 (lanes 1−6) and T_3RE-MoMLV (lanes 7−12) and ΔA-RARβ extracts (lanes 2 and 8), p24-RARβ (lane 6), control extracts (lanes 1 and 7), p46-T_3R (lane 12), and extracts containing various ratios of ΔA-RARβ and p24-RARβ (lanes 3−5) or ΔA-RARβ and p46-T_3R (lanes 9−11). The T_3R complexes I, II and III and the p46-T_3R−ΔA-RARβ heterodimer complex (arrow) are indicated, the filled circle indicates a non-specific complex. HeLa monolayer cells were infected with recombinant vaccinia virus expressing various T_3Rs and RARs. Extract preparation, gel retardation assay and oligonucleotides are described in Materials and methods.

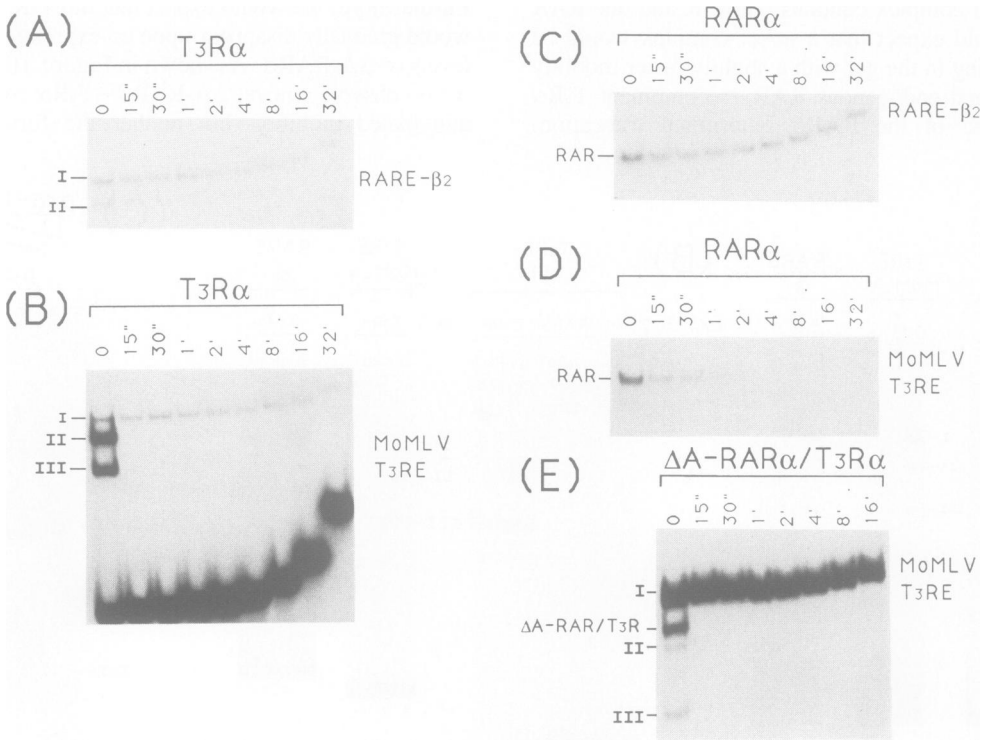

Fig. 2. Stability of protein–DNA complexes in off-rate experiments. Receptor containing extracts were mixed with ^{32}P-labelled oligonucleotides and protein–DNA complexes were allowed to form. A large excess of unlabelled oligonucleotide was added at time 0 and aliquots were loaded on polyacrylamide gels at the indicated time points. (**A**) Off-rate of $T_3R\alpha$ complexes I and II on RARE-β2. (**B**) Off-rate of $T_3R\alpha$ complexes I, II and III on MoMLV-T_3RE. (**C**) Off-rate of RARα on RARE-β2. (**D**) Off-rate of RARα on T_3RE-MoMLV. (**E**) Off-rate of T_3R–RAR heterodimer on T_3RE-MoMLV. The experiments were performed as described in Materials and methods.

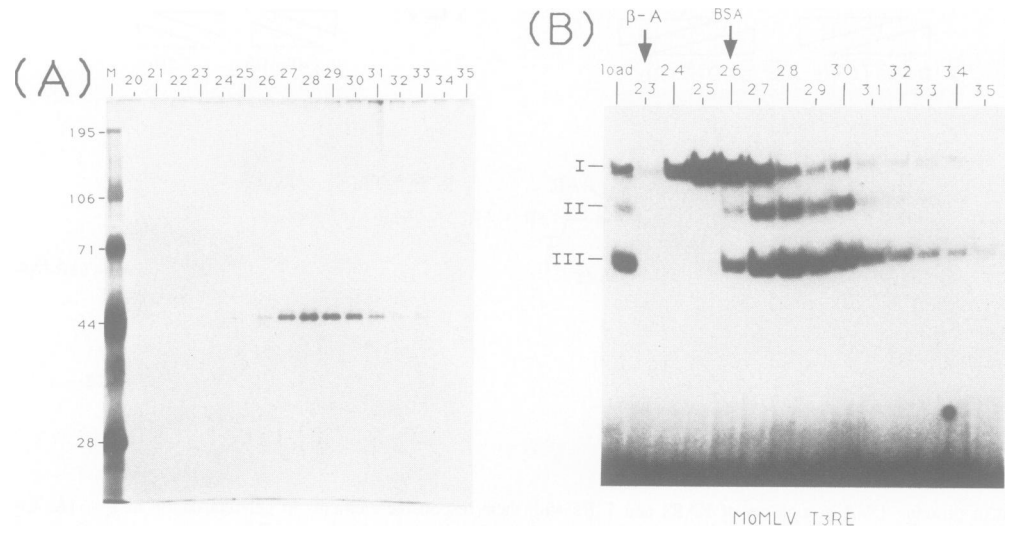

Fig. 3. Copurification of X with T_3R. His-T_3R purified on NTA and S12 columns was passed over an FPLC S12 gel filtration column. (**A**) Silver stained SDS–polyacrylamide gel of column fractions. The size of molecular weight markers is indicated. (**B**) Gel retardation assay of corresponding S12 column fractions using the T_3RE-MoMLV as labelled oligonucleotide. The load contained 1/10 of the volume used for assaying the fractions. Complexes I, II and III are indicated. Arrows labelled β-A and BSA indicate the positions of β-amylase and BSA in the S12 column.

abundance of the T_3R type I complex was affected by co-expression of the ΔA-RARβ protein. It should be noted that the ΔA-RARβ was expressed at a lower level than $T_3R\alpha$ and did not form a detectable complex with the MoMLV-T_3RE at this receptor concentration (Figure 1E, lane 8).

We conclude that the type I $T_3R\alpha$ complex does not consist of an RAR–$T_3R\alpha$ heterodimer but of $T_3R\alpha$ complexed with an unidentified auxiliary factor ('X'). Furthermore, the T_3R–'X' and T_3R–RAR complexes migrate to the same position in the gel suggesting that the auxiliary factor has a molecular weight comparable to that of RAR, i.e. ~55 kDa. Similarly, the RAR complex that specifically binds to the RARE-β_2 also consists of an RAR and an auxiliary factor.

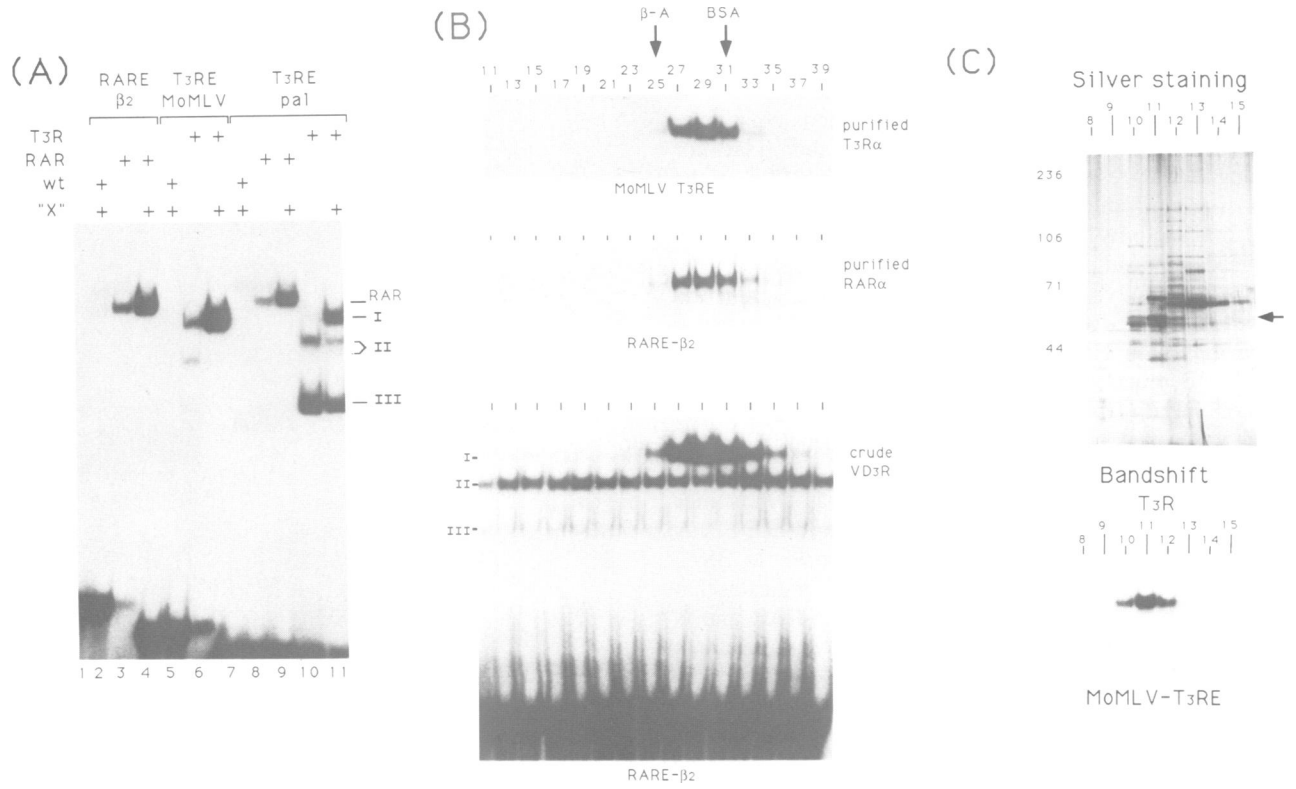

Fig. 4. Identity of RAR 'X' and T_3R 'X'. (**A**) Gel retardation assays were performed with oligonucleotides RARE-β2 (lanes 1–3), T_3RE-MoMLV (lanes 4–6) and T_3RE-pal (lanes 7–11) and extracts containing RARα (lanes 3, 4, 8 and 9), T_3Rα (lanes 5, 6, 10 and 11) or control extracts (lanes 1, 4 and 7) without (lanes 2, 5, 8 and 10) or with (lanes 1, 3, 4, 6, 7, 9 and 11) addition of the 'X' fraction. (**B**) Highly enriched 'X' protein was subjected to gel filtration on an FPLC S12 column. Column fractions were mixed with purified T_3Rα (top panel), purified RARα (middle panel) or VD_3R containing extract (bottom panel) and gel retardation assays were performed using the indicated oligonucleotides. Arrows labelled β-A and BSA indicate the position of β-amylase and BSA in the S12 column. (**C**) Silver stained SDS–polyacrylamide gel of fractions from Mono-Q column. Arrow indicates the position of the protein comigrating with the complementing activity. Position of molecular weight markers is indicated. Bandshift complementation with corresponding fractions using T_3Rα extract and the T_3RE-MoMLV oligonucleotide is shown below.

RAR–'X' and T_3R–'X' complexes are stably bound to DNA

The apparent affinity of the various receptor–DNA complexes was assessed by off-rate experiments challenging a preformed complex with an excess of cold specific probe and applying aliquots at different times after addition of the competitor onto a running gel. As shown in Figure 2B, the type I complex (T_3R–'X') bound to the MoMLV-T_3RE is stable upon challenge whereas the type II (2×monomer) and type III (monomer) complexes immediately disappear upon addition of the cold competitor. Similar results were obtained using the T_3RE-pal probe. The RAR–'X' complex is also stably bound to the RARE-β_2 and is not decreased significantly upon addition of the cold competitor (Figure 2C). The low level of T_3R–'X' complex bound to the RARE-β2 probe is stable upon challenge (Figure 2A) whereas the RAR–'X' complex is not very stably bound to the MoMLV-T_3RE (Figure 2D). Finally, we assessed the stability of the RAR–T_3R complexes. In order to distinguish between the T_3R–'X' and the RAR–T_3R complexes in bandshift assays, we exploited the observation made in the double infection experiments shown in Figure 1E, lanes 8–12. Again, the T_3R–'X' complex (type I) is stable whereas the ΔA-RAR–T_3Rα complex is highly unstable and is abolished upon competition (Fig 2E). Also, full-length RAR–T_3R complexes were completely unstable in off-rate experiments (data not shown). These results substantiate our conclusion that an auxiliary factor is required for stable DNA binding of T_3R and RAR. Furthermore, RAR and T_3R can form a heterodimer in the presence of DNA, but the ternary complex is very unstable.

The auxiliary factor(s) is tightly associated with RAR and T_3R

Rapid and quantitative binding of proteins can be facilitated through N-terminal tagging of the protein with histidines and subsequent purification using a Ni^{2+}-chelate NTA column (Hochuli *et al.*, 1988; Gentz *et al.*, 1989). Binding of the protein and elution under native conditions can be achieved using imidazole as the eluent (Janknecht *et al.*, 1991). Because of the mild purification conditions, it should be possible to co-purify proteins associated with the His-tagged receptor. We addressed the question whether or not the T_3R–'X' and RAR–'X' protein complexes are formed in solution in the recombinant virus infected cell and, if so, whether the auxiliary factor(s) co-purifies with the His-tagged receptors. Nuclear extracts were prepared from cells infected with recombinant viruses expressing either His-tagged T_3Rα or RARα receptors, loaded on to the NTA column and developed with increasing concentration of imidazole. The receptors were subsequently purified using an FPLC Mono-S and S12 gel filtration columns. The His-T_3Rα and His-RARα now comprise >95% and 30%, respectively,

of the protein detectable by silver staining (Figure 3A and data not shown). The vast majority of receptor elutes from the S12 column later than BSA, as anticipated for a monomeric receptor.

Next, we analysed the S12 column fractions containing His-tagged $T_3R\alpha$ and RARα in gel mobility shift assays for binding to their responsive elements. More than 90% of the total $T_3R\alpha$ (Figure 3B) and RAR (data not shown) bandshift activity was recovered as determined by quantification using a Molecular Dynamics Phosphoimager. More importantly, the type I complex ($T_3R\alpha$–'X') was quantitatively recovered, indicating that the auxiliary factor co-purifies with His-$T_3R\alpha$. The $T_3R\alpha$–'X' heterodimer (Figure 3B, fractions 24–28) elutes from the column prior to monomeric His-$T_3R\alpha$ (Figure 3B, fractions 26–31) which yields the type II and III complexes in bandshift assays in agreement with our previous assessment. Similarly, the vast majority of purified His-RARα eluted as a monomer from the S12 column and did not bind to the RARE-β2. A small proportion of the His-RARα eluted prior to BSA, giving rise to the RAR specific bandshift. This indicates that the auxiliary factor is tightly associated with RAR and is essential for the binding of RAR to its responsive element (data not shown).

Several conclusions can be drawn from these experiments. Firstly, the auxiliary factor(s) co-purifies with a small proportion of the overexpressed His-tagged receptor. Secondly, the receptor–auxiliary factor interaction is highly stable in solution. Thirdly, the vast majority of receptor is monomeric in solution and yields no or only unstable complexes in bandshift assays. Fourthly, >95% of the receptor is monomeric, suggesting that the auxiliary factor is titrated out due to overexpression of the receptor in recombinant vaccinia virus infected cells.

T_3R, RAR and VD_3R associate with an identical auxiliary factor

We next analysed whether addition of nuclear extracts from uninfected HeLa cells could augment the formation of a stable receptor–DNA complex. The addition of protein fractions enriched in auxiliary factor activity to crude extracts of HeLa cells infected with T_3R or RAR strongly enhances the formation of the RAR and T_3R type I complexes (Figure 4A). Addition of auxiliary factor in bandshift assays using T_3R extracts results not only in an enhanced formation of the type I complex, but also in a simultaneous reduction in the amount of the type II and III complexes (compare lanes 5 and 10 with 6 and 11). The auxiliary factor alone is unable to bind at this concentration to either of the three oligonucleotides used throughout this study (lanes 1, 4 and 8). Off-rate experiments showed that the newly formed receptor–auxiliary factor complexes bind to their cognate binding sites with high affinity indistinguishable from the purified T_3R–'X' and RAR–'X' protein complexes (data not shown). These experiments demonstrate that the auxiliary factor(s) purified from HeLa cells is capable of associating with the large pool of monomeric T_3R and RAR protein as found in recombinant vaccinia virus infected HeLa cell extracts yielding the stable DNA binding complex.

Surprisingly, the auxiliary factor activities for RAR and T_3R co-elute from four different columns, suggesting that they are very similar if not identical proteins. To establish the identity of the auxiliary factor(s) further, the protein

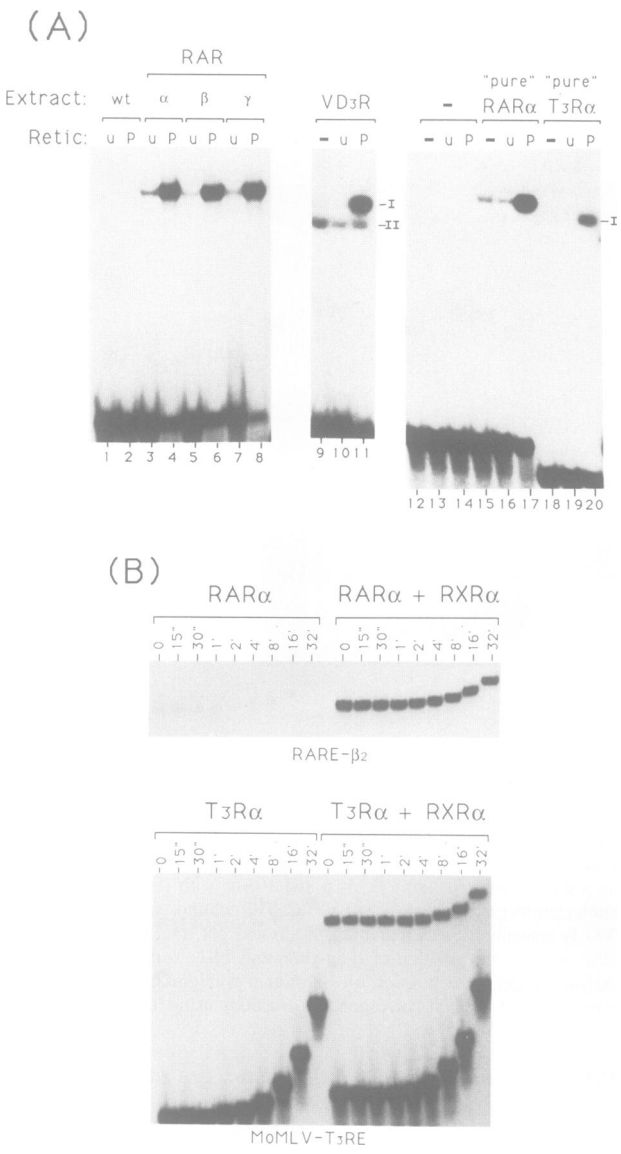

Fig. 5. RXRα substitutes functionally for 'X'. (**A**) Gel retardation assays performed with RARE-β2 (lanes 1–17) or T_3RE-MoMLV (lanes 18–20) using control extracts (lanes 1 and 2), extracts containing RARα (lanes 3 and 4), RARβ (lanes 5 and 6), RARγ (lanes 7 and 8) and VD_3R (lanes 9–11) or purified RARα and $T_3R\alpha$. The extracts and purified proteins were complemented with unprogrammed reticulocyte lysate (indicated by U) or lysate programmed with *in vitro* transcribed RXRα mRNA (indicated by P). (**B**) Stability of RXRα complexes. Off-rates were performed as described in Figure 2 with RARα extract on RARE-β2 and $T_3R\alpha$ extracts on MoMLV-T_3R not complemented or complemented with *in vitro* translated RXRα as indicated.

fraction purified over four columns was subsequently separated on an FPLC S12 gel filtration column. Aliquots of the different fractions were tested for their ability to form a complex with semi-pure RAR and T_3R monomers. The auxiliary factor(s) complementing T_3R and RAR bandshift activities co-elute from the column, peaking in fractions 27–31 (Figure 4B). We could not detect protein–DNA complexes in these fractions in the absence of added $T_3R\alpha$ or RARα (data not shown).

Finally, we assayed whether binding of the closely related VD_3R could be enhanced by this auxiliary factor preparation. Recombinant VD_3R expressed via the vaccinia

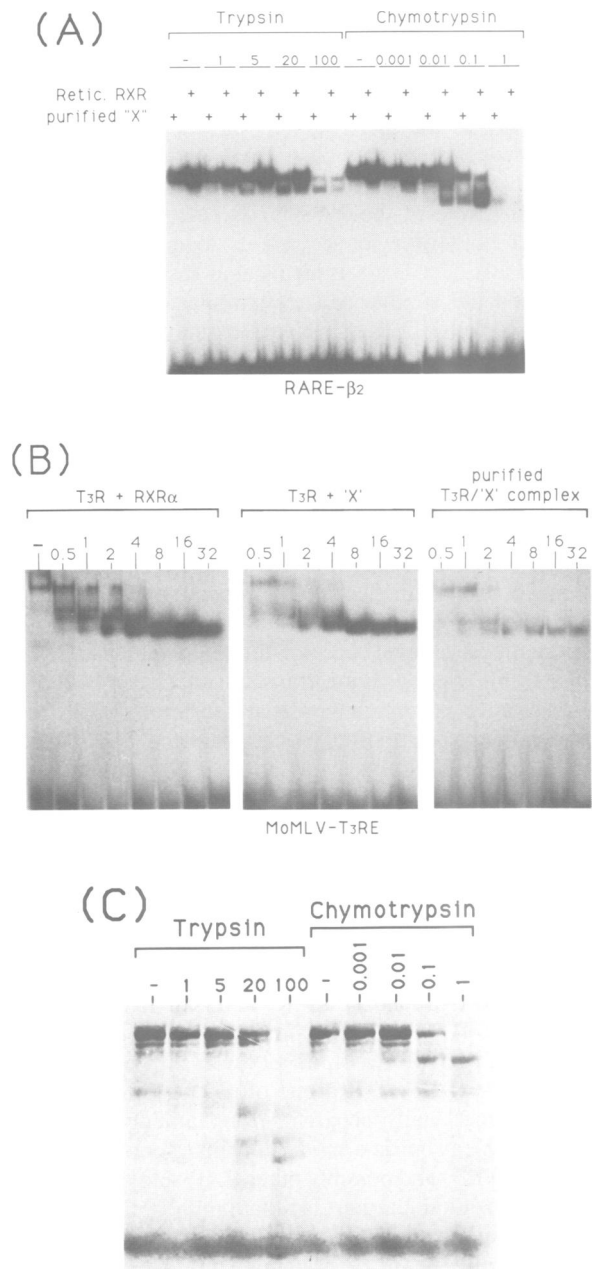

Fig. 6. Identical proteolytic cleavage pattern of receptor complexes. (**A**) RARα containing HeLa cell nuclear extracts and RARE-β2 were mixed and supplemented with either 'X' fraction or reticulocyte produced RXRα as indicated. Trypsin and chymotrypsin were added as indicated (ng per bandshift reaction) and after 10 min incubation at 20°C, the complexes were loaded on bandshift gels. (**B**) Clipping assay performed with T_3Rα extracts mixed with either RXRα. or 'X' and T_3R−'X' complex obtained from the S12 column. Dispase I added as indicated (mg). (**C**) Proteolytic clipping of [^{35}S]methionine-labelled, *in vitro* translated RXRα. Aliquots of the bandshift fraction after digestion with trypsin and chymotrypsin (shown in panel A) were separated on a 10% SDS−polyacrylamide gel.

system binds relatively strongly to the RARE-β_2, but very poorly to the MoMLV-T_3RE probe (T.H.Bugge and H.G.Stunnenberg, unpublished observations). As shown in Figure 4B, one major (II) and two minor complexes (I and III) can be detected in bandshift assays. Auxiliary factor activity can be detected, peaking in fractions 27−31, yielding VD_3R type I complexes in bandshift assays.

Taken together, these findings strongly suggest that one and the same auxiliary factor associates with T_3Rα and RARα and VD_3R. Inspection of the protein pattern after SDS−PAGE and silver staining of the fractions from the Mono-Q column revealed a protein of ~55 kDa which co-elutes with the auxiliary factor activity (Figure 4C). This is in good agreement with the estimated molecular weight of the auxiliary factor based on the mobility of the complexes in bandshift experiments.

The auxiliary factor is indistinguishable from RXRα

Several observations suggest that the auxiliary factor could be a member of the T_3R−RAR receptor subfamily. Firstly, the auxiliary factor stably associates with RAR, T_3R and VD_3R via the dimerization interface in the C-terminal half of the receptors (J.Pohl and H.G.Stunnenberg, unpublished data). Thus, the auxiliary factor is likely to have a related major dimerization interface which is a hallmark of the nuclear receptor family. Secondly, binding of RAR−'X' to the RARE-β_2 element requires both direct half-site repeats because mutations in either one of the repeat elements abolish binding (Vivanco Ruiz *et al.*, 1991; Sucov *et al.*, 1990). The same holds for the binding of T_3R−'X' to the MoMLV-T_3RE, suggesting that the auxiliary factor has half-site sequence requirements for binding identical to those of members of this subfamily (G^G_TTCA). Three additional findings point to the retinoid X receptor α, RXRα, as an auxiliary factor candidate. Firstly, RXRα has a mol. wt of 54 kDa, which is similar to that of the auxiliary factor. Secondly, RXRα, has been reported to stimulate RA dependent transcription in a cell line containing high levels of endogenous RAR (Mangelsdorf *et al.*, 1991). Furthermore, the auxiliary factor was unable to augment binding of RXRα to a reported RXR binding element (RXRE) (J.Pohl, T.H.Bugge and H.G.Stunnenberg, unpublished observations) from the CRBP II promoter (Mangelsdorf *et al.*, 1991). We therefore tested whether RXRα could substitute functionally for the purified auxiliary factor to facilitate high affinity DNA binding of T_3R, RAR and VD_3R. For this purpose, RXRα cDNA was transcribed and translated *in vitro* in a rabbit reticulocyte lysate. RXRα programmed and unprogrammed reticulocyte lysates were added to recombinant RAR (α, β or γ) containing crude extracts or control extracts from wild-type virus infected cells. As shown in Figure 5A, RXRα protein containing lysates strongly enhanced binding of RAR to the RARE-β_2 probe. Addition of *in vitro* translated RXRα to crude VD_3R containing extracts resulted in the formation of type I complexes using the RARE-β_2 probe (Figure 5A lanes 9−11). Addition of RXRα containing lysates to highly purified His-RARα and His-T_3Rα protein also facilitated the formation of a complex migrating at the same position as the purified auxiliary factor facilitated complexes (Figure 5A, lanes 12−20). Off-rate experiments showed that the RARα−RXRα and T_3Rα−RXRα complexes are stable (Figure 5B). These data indicate that RXRα can substitute functionally for the purified auxiliary factor in generating a stable receptor−DNA complex migrating identically to the RAR−'X' and T_3R−'X' complexes. The receptor−RXRα complexes were formed and stable in the absence or presence of ligands (data not shown).

To establish unequivocally the identity of the purified auxiliary factor as RXRα, we performed clipping bandshift

experiments (Schreiber *et al.*, 1988; Schöler *et al.*, 1990). Preformed DNA–protein complexes generated by crude extracts containing RARα and purified 'X' or reticulocyte translated RXRα and the RARE-β_2 probe were treated with trypsin or chymotrypsin prior to loading on to bandshift gels (Figure 6A) Identical degradation products were obtained using either 'X' or recombinant RXRα. Preformed DNA–protein complexes generated by purified His-T_3Rα–'X' (fraction 24 in Figure 3) or complementation of purified His-T_3Rα monomers (fraction 34 in Figure 3) with either purified auxiliary factor (fraction 29 in Figure 4) or recombinant RXRα were treated with Dipase I (Figure 6B), trypsin and chymotrypsin (data not shown) prior to loading on to bandshift gels. Similarly, the clipping pattern of the various His-T_3R based complexes generated by the dispase I enzyme were identical. We confirmed the enzymatic digestion of the [^{35}S]methionine-labelled RXRα part of the heterodimers by SDS–PAGE of bandshift aliquots (Figure 6C).

In conclusion, RXRα is indistinguishable from the purified auxiliary factor by DNA binding and biochemical criteria.

Discussion

We have investigated the protein–protein and protein–DNA interactions of the T_3R–RAR group of nuclear receptors. By biochemical analyses a small proportion of overexpressed T_3R and RAR was found to be associated with a nuclear protein in solution. The interaction of T_3R and RAR with this protein is extremely stable as the protein co-purified through five different columns and in the presence of non-ionic detergents. The auxiliary factor associating with T_3R and RAR was found to be an identical protein by purification. This protein also facilitated the formation of a VD_3R–DNA complex, indicating that the auxiliary factor associates with all three receptors. Heterodimerization of T_3R and RAR with the auxiliary protein was found to be required for stable interaction of the receptors with their responsive elements. The vast majority of overexpressed T_3R and RAR was found to be monomeric as judged from its migration in gel filtration columns. The monomeric RAR did not demonstrate appreciable binding to the RARE-β_2, and monomeric T_3R yielded highly unstable complexes with direct or inverted repeat type T_3 responsive elements. The presented data indicate that these receptors, unlike the steroid hormone receptors, do not form stable homodimers in solution, or interact with their responsive elements as homodimers. Under the experimental conditions used, the recombinant T_3R and RAR are highly overexpressed and titrate out the auxiliary factor activity. It is likely that the majority of T_3R, RAR and most likely the VD_3R is normally found complexed with the auxiliary factor within the cell. The functional properties of the auxiliary factor described here match well the properties of various activities in nuclear extracts reported to enhance binding of T_3R (Murray and Towle, 1989; Burnside *et al.*, 1990; Darling *et al.*, 1991), RAR (Glass *et al.*, 1990) and VD_3R, respectively (MacDonald *et al.*, 1991; Sone *et al.*, 1990). Thus our data indicate that these activities may be caused by a single or closely related protein(s) identical to the auxiliary factor identified in this study.

We found that overexpressed RAR and T_3R form heterodimers on T_3RE-pal and T_3RE-MoMLV. This finding is in accordance with data published by Glass and coworkers (Glass *et al.*, 1989). However, the heterodimer was highly unstable on these responsive elements indicating a low affinity of the complex. Because of the prominent shift obtained with the heterodimer on the T_3RE-MoMLV element which indicates that RAR and T_3R bind in a cooperative manner to this element, we cannot fully exclude a functional role of the RAR–T_3R heterodimer.

Several lines of evidence led us to conclude that our identified auxiliary factor is identical to RXRα. Firstly, both RXRα and the auxiliary factor stimulated the binding of RAR, T_3R and VD_3R to DNA. Secondly, the complexes formed between the receptors and RXRα migrated to an identical position to those formed between the three receptors and the auxiliary factor as obtained after extensive purification. Thirdly, RAR and T_3R formed very stable complexes with both RXRα and the auxiliary factor on their responsive elements. Fourthly, proteolytic clipping bandshifts using three different proteases revealed identical patterns with purified complexes or *in vitro* reconstituted complexes using purified 'X' or recombinant RXRα.

This finding is rather surprising since RXRα has been proposed to be a novel receptor for retinoids (Mangelsdorf *et al.*, 1990). Furthermore, transactivation mediated by an RA responsive element in the CRBPII promoter (RXRE) has been reported to be boosted by cotransfected RXRα but not RAR in EC cells (Mangelsdorf *et al.*, 1991). In our hands, vaccinia produced or *in vitro* translated RXRα failed to demonstrate an appreciable binding to this RXRE, but the binding of RXRα to the element was stimulated by addition of RAR. (J.Pohl, T.H.Bugge and H.G.Stunnenberg, unpublished observations). Further work is needed to clarify these points.

With RXRα being a heterodimerization partner of RAR, T_3R and most probably VD_3R, and with the absence of homodimeric RAR and T_3R complexes in solution or stable homodimeric complexes of the receptors on their responsive elements, the RAR–T_3R group of receptors clearly stands out as a functionally separate group among the nuclear receptors. We propose a model for this group of receptors in which RXRα (and possibly other RXR subtypes) function as a dimerization partner facilitating the binding of the various ligand responsive receptors to their responsive elements. In the light of previous studies on RA and T_3 responsive elements, we conclude that RXRα binds in a sequence specific manner to one of the two half-sites comprising the responsive elements. Indeed, the P-box of RXRα is identical to the T_3R–RAR type, and, thus, the predicted half-site sequence preference is GGTCA. As RXRα is common to the dimeric receptor complexes, the spacing requirements of RAR and T_3R for binding to and transactivating through specific responsive elements (Vivanco Ruiz *et al.*, 1991; Näär *et al.*, 1991; Umesono *et al.*, 1991) must be determined by features within the T_3R and RAR. Work is in progress to clarify this point.

We have shown that T_3Rs and RARs form heterodimers but not homodimers in solution and stably bind their cognate responsive elements as heterodimers. This may solve a conceptual problem arising from the existence of multiple T_3R and RAR subtypes and isoforms often being coexpressed within a single cell. The various subtypes and isoforms are believed to respond differentially to ligand, thereby enabling the pleiotropic effects of RA and T_3

(Leroy *et al.*, 1991; Zelent *et al.*, 1991; Forrest *et al.*, 1990, 1991). The many subtypes and isoforms differ mainly in their N- and C-termini, while their DNA binding and dimerization domains are virtually identical. If RAR and T_3R functioned as homodimers, unrestricted dimerization between the subtypes and isoforms would be the predicted consequence. It is difficult to envision how such promiscuous dimerization would allow each subtype and isoform to exert its individual function(s).

In conclusion, we have shown that RARs, T_3Rs and probably VD_3R are tightly associated with RXRα. The heterodimerization with RXRα is both necessary and sufficient for stable interaction of the receptors with their responsive elements. Further work is required to establish the consequences of these findings for our understanding of the mechanism of action of retinoids, vitamin D_3 and thyroid hormones.

Materials and methods

Recombinant viruses and preparation of extracts from virus infected cells

Recombinant vaccinia virus expressing RARα, β or γ, T_3Rs, VD_3R and RXRα as well as modified forms of the receptors were constructed and amplified using standard procedures (de Magistris and Stunnenberg, 1988; Stunnenberg *et al.*, 1988; Janknecht *et al.*, 1991). Preparation of nuclear extracts from virus infected cells was performed as described (Vivanco Ruiz *et al.*, 1991).

Gel retardation assays and proteolytic clipping gel retardation assay

Oligonucleotides were synthesized with 5′ extensions and annealed double stranded oligonucleotides were labelled by filling in with [^{32}P]dCTP and Klenow DNA polymerase. Nuclear extract was incubated in a binding buffer containing 15% glycerol, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 50 mM KCl, poly(dI−dC) (0.2 mg/ml final concentration) and 0.01% Triton X-100 for 15 min on ice. For bandshifts with purified proteins, binding buffer was supplemented with 5 mg/ml BSA. Subsequently, 5−40 fmol of probe was added and the binding reaction was continued for a further 15 min. Off-rate assays were performed by adding a 500- to 1000-fold excess of specific oligonucleotides to the preformed protein−DNA complexes. Complexes formed during the bandshift reactions were separated on pre-cooled, pre-run (1 h, 200 V) 6% polyacrylamide gels containing 0.25×TBE at 4°C at 200 V, dried and exposed. For the proteolytic clipping assay, binding buffer was supplemented with BSA to a total protein concentration of 0.25 mg/ml. One microlitre of freshly diluted protease was added to the reaction mixture and allowed to digest for 10 min at 20°C. Concentrations per reaction were 0.5−32 μg of dispase I (Boehringer Mannheim), 1−100 ng of trypsin (Promega) and 0.001−1 ng of chymotrypsin (Promega).

Oligonucleotides

The following oligonucleotides and their complements were used in the study: RARE-β2: 5′-TCGACGGGTAGGGTTCACCGAAAGTTCACTCGC-3′; T_3RE-MoMLV: 5′-TCGACAGGGTCATTTCAGGTCCTTGC-3′; T_3RE-pal: 5′-AGCTTCAGGTCATGACCTGAAGCA-3′; and RXRE: 5′-AGCTGTCACAGGTCACAGGTCACAGGTCACAGTTCAAGCT-3′.

Purification of the HeLa auxiliary factor

Nuclear extract was prepared from 100 l of HeLa S3 spinner cells according to Dignam *et al.* (1983). $(NH_4)_2SO_4$ was added up to 0.8 M final concentration and the extract was passed over a phenyl-FF (Pharmacia) column equilibrated with S-buffer [20 mM HEPES−KOH (pH 7.8), 17% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT] supplemented with 0.8 M $(NH_4)_2SO_4$. The bound proteins were eluted with a 800−10 mM $(NH_4)_2SO_4$ gradient. The 300−10 mM fractions containing the auxiliary factor activity were pooled and loaded onto a Ni^{2+}-NTA column and bound proteins were eluted with two column volumes of S-50 buffer (S-buffer containing 50 mM NaCl plus 0.1% Triton X-100) supplemented with increasing concentrations of imidazole (1, 13 and 25 mM respectively). Fractions containing the auxiliary factor activity were pooled and applied onto an FPLC Mono-S column; bound proteins were then eluted with a 50−500 mM NaCl gradient in S-buffer containing 0.1% Triton X-100. Fractions containing the complementing activity eluting between 250 and 310 mM NaCl were pooled and applied onto a Mono-Q column (Pharmacia) and developed with a 50−500 mM NaCl gradient in S-buffer containing 0.1% Triton X-100. The complementing activity eluted between 50 and 100 mM NaCl. Fractions containing the auxiliary factor activity were pooled, concentrated and loaded onto an FPLC S12 sieving column. In a parallel run, the protein markers (BSA and β-amylase) were applied. Resulting fractions were tested in gel retardation assays for complementation of receptors and were analysed by SDS−PAGE and silver staining.

Purification of histidine-tagged receptors

Nuclear extracts were prepared from 15 litres of HeLa S3 spinner cells infected with either His-T_3Rα or His-RARα virus. The extracts were applied onto a Ni^{2+}NTA column and the bound protein was eluted with imidazole as described (Janknecht *et al.*, 1991). Eluted material was applied onto a Mono-S column and the column was developed with a 50−1000 mM NaCl gradient in S-buffer. The peak fractions containing the receptors were applied onto an S-12 gel filtration column and the column was developed with S-buffer containing 500 mM NaCl and 0.1% Triton X-100.

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