Vol. 175, No. 1, 1991
February 28, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 250-255

Zn^{+2} INDUCES REVERSIBLE CROSS-LINKING OF HUMAN PLACENTAL THYROID HORMONE NUCLEAR RECEPTOR WITH NO EFFECT ON HORMONE BINDING

Kwang-Huei Lin and Sheue-Yann Cheng*

Laboratory of Molecular Biology, National Cancer Institute
National Institutes of Health, Bethesda, MD 20892

Received January 3, 1991

Zn^{+2} is required for specific binding of c-*erb*A proteins to the hormone response elements of target genes. It is unclear whether Zn^{+2} is important for the binding of ligand to c-*erb*A proteins. The present study evaluated the effect of Zn^{+2} and other divalent cations on the binding of 3,3',5-triiodo-L-thyronine(T_3) to the purified human placental c-*erb*A protein (h-TRβ1). Zn^{+2} induced cross-linking of h-TRβ1 to form aggregates in a dose-dependent manner with an apparent half-maximal concentration of ~200 μM at 22°C. Cross-linking was reversible by the addition of 5 μM EDTA or 10 mM dithiothreitol. The cross-linked h-TRβ1 bound T_3. These results indicated Zn^{+2} had no effect on T_3 binding and suggested that the cysteines and histidines involved in cross-linking are not essential for T_3 binding.

The thyroid hormone 3,3',5-triiodo-L-thyronine (T_3) promotes growth, induces differentiation and regulates metabolic and developmental processes. Studies on the mechanisms of thyroid hormone action have been facilitated by the isolation of cDNAs for thyroid hormone nuclear receptors (1,2). By analogy with steroid hormone/retionic acid receptors, the regulation of gene expression was thought to be mediated through the interaction of the DNA binding domain of receptors with the specific DNA sequences of responsive genes. Recently the solution structure of the DNA binding domain of the glucocorticoid receptor has been determined by nuclear magnetic resonance spectroscopy and distance geometry (3). The DNA binding domain has finger-like structures with two zinc ions forming a tetra-hedral coordination complex with cysteine residues. Thus, Zn^{+2} is required for the proper folding and for specific binding to the hormone-responsive DNA sequences of target genes (3,4). However, whether Zn^{+2} is also important for hormone binding is unclear. Recently, Surks *et al.* have demonstrated that the binding of T_3 to

*To whom correspondence should be addressed.

Abbreviations: T_3, 3,3',5-triiodo-L-thryonine; h-TRβ1, human placental thyroid hormone nuclear receptor: DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

0006-291X/91 $1.50

nuclear extracts of GC cells was inhibited by Zn^{+2} and other divalent cations (5). The inhibition was proposed to occur by formation of Zn^{+2}-induced inhibition of T_3 binding to nuclear receptors. Furthermore, Lu *et al.* reported that the binding of T_3 to the *in vitro* translated rat c-*erb*Aβ receptor was also inhibited by Zn^{+2} with a half-maximal concentration of 50-100 μM (6).

We have recently expressed large amounts of h-TRβ1 in *E. coli* and purified it to apparent homogeneity (7). The purified h-TRβ1 binds to T_3 and its analogs with affinity and specificity similar to those reported for the T_3 nuclear receptors identified in tissues and cultured cells. Using affinity labeling with [3',5'-^{125}I]-L-thyroxine, the hormone binding site was identified to be in a domain beginning at Phe_{240} and ending at Asp_{456} (7). Sequence analysis indicates that a cluster of cysteines and histidines (His_{400}-His_{436}) are located near the carboxyl terminus of the hormone binding domain. Analysis of the distribution pattern of histidines and cysteines identified the sequences His_{408}-X_2-His_{411} and His_{436}-X_4-Cys_{441} which are the Zn^{+2} binding sites in Cu-Zn superoxide dismutase (E.C 1.15.11) and Cu^{+2} binding site in azurin, respectively (8). The motif of His_{408}-X_2-His_{411}-X_{17}-Cys_{429}-$His4_{30}$-X_4-$His4_{36}$ in h-TRβ1 is very similar to the putative zinc-finger sequences found in Phi-X174 gene A protein, G_4 gene A protein and T_4 DNA primase (8). We, therefore, evaluated whether Zn^{+2} and receptors form complexes and, furthermore, if such complexes are formed, whether binding of T_3 would be affected. We studied the effect of Zn^{+2} and other divalent cations on T_3 binding to the purified h-TRβ1. We found that Zn^{+2} induced cross-linking of h-TRβ1 to form aggregates. The formation of aggregates was reversible by the addition of EDTA and DTT. Complexing of h-TRβ1 with Zn^{+2} did not inhibit T_3 binding.

MATERIALS AND METHODS

Materials - [3'-^{125}I]T_3 (2200 Ci/mmol, 1 Ci = 37 GBq) was purchased from DuPont/New England Nuclear. Isopropyl-β-D-thiogalactopyranoside was from Research Organics, Inc. (Cleveland, OH). Dithiothreitol, $ZnCl_2$, $CdCl_2$, $CoCl_2$, $MnCl_2$, and $NiCl_2$ were purchased from Sigma. Sephadex G-25 (fine) was obtained from Pharmacia. Dowex 1-X8 was from BioRad Co.

Preparation of $ZnCl_2$ and other divalent cation solutions - The divalent cation solutions were freshly prepared on the day of experiments. Stock solutions (100 mM) were prepared in H_2O. Subsequent serial dilutions were made in water. Five μl of water solution was added to the assay mixture to give the final concentrations stated in the Legends for various purposes.

Expression and purification of h-TRβ1 - h-TRβ1 was expressed and purified from *E. coli* by methods similar to that described by Lin and Cheng (7), except that h-TRβ1 obtained after renaturation was used directly for T_3 binding studies. The purity of h-TRβ1 was greater than 90%. The h-TRβ1 solution was stored at -70°C for months without loss of T_3 binding activity.

Effect of Zn^{+2} and other divalent metal ions on the binding of T_3 to h-TRβ1 - On the day of the experiment, frozen h-TRβ1 was thawed and dialysed

against 500 ml of buffer B (50 mM Tris-HCl/8.0, 0.2 M NaCl, 0.01% Lubrol, 10% glycerol and 1 mM DTT) for 3 hrs at 4°C with one change of dialysate. Ten µl of highly purified h-TRβ1 (10-20 µg/ml) or *E. coli* lysate containing the expressed h-TRβ1 (7) was incubated with 0.2 nM of [^{125}I]T_3 in 240 µl of buffer B for 60 min at 22°C. $ZnCl_2$ or other divalent cations (5 µl) in various concentrations was added for 60 min. After incubation, the [^{125}I]T_3 bound to the soluble form of h-TRβ1 was separated from unbound [^{125}I]T_3 and aggregated h-TRβ1 by using G-25 (fine) column chromatography (9,10). The aggregated h-TRβ1 was retained on the top of column and, therefore, separated from the soluble form of h-TRβ1. In some experiments, we also used a Dowex 1-X8/400 mesh anion exchange resin method (11) to separate the aggregated h-TRβ1 and unbound [^{125}I]T_3 from the soluble form of [^{125}I]T_3-h-TRβ1. To quantify the aggregated h-TRβ1-bound to [^{125}I]T_3, the incubation mixture was centrifuged at 11,000 xg for 15 min at 4°C and the pellet was counted. The amounts of soluble h-TRβ1 were also analyzed by a 10% SDS-PAGE. In some experiments, 10 µl of h-TRβ1 (10-20 µg/ml) was first incubated with $ZnCl_2$ in various concentrations for 30-60 min at 22°C. [^{125}I]T_3 was added to give a final concentration of 0.2 nM. After further incubation, the analysis of soluble h-TRβ1 and aggregated h-TRβ1 was carried out as described above.

RESULTS AND DISCUSSION

To evaluate whether Zn^{+2} complexed with the h-TRβ1 to form aggregates, we first determined if the soluble form of h-TRβ1 bound to [^{125}I]T_3 by passing the incubation mixture through a G-25 column. Figure 1 shows that the [^{125}I]T_3 bound to the soluble form of h-TRβ1 was reduced by Zn^{+2} in a dose-dependent manner with an apparent half-maximal concentration of ~200 µM. Figure 1 also shows that Co^{+2}, Cd^{+2}, and Ni^{+2} are more effective than Zn^{+2} in

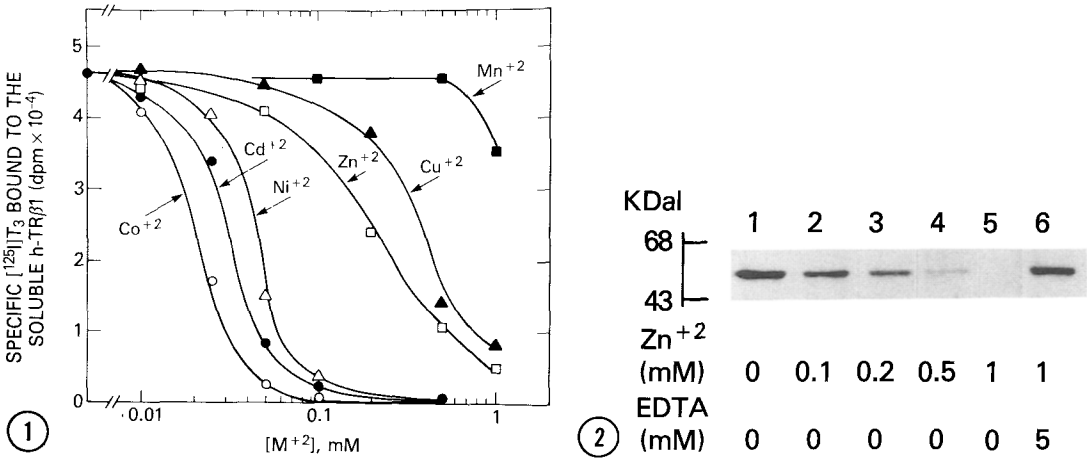

Figure 1. Recovery of soluble form of h-TRβ1 in the presence of increasing concentrations of divalent metal cations.
h-TRβ1 (0.1 µg/0.25 mL) in buffer B was incubated with 0.2 nM [^{125}I]T_3 for 60 min at 22°C. Five µl of freshly prepared $CoCl_2$ (○), $CdCl_2$ (●), $NiCl_2$ (Δ), $ZnCl_2$ [□], $CuCl_2$ (▲), or $MnCl_2$ (■) was added. After incubation for an additional 60 min at 22°C, [^{125}I]T_3 bound to soluble h-TRβ1 was determined as described in Methods.

Figure 2. The soluble fraction of h-TRβ1 as analyzed by SDS-PAGE.
h-TRβ1 (0.16 µg/50 µL) in buffer B was incubated with 0.2 nM T_3 in the presence of increasing concentration of $ZnCl_2$ (5 µL) for 1 hr at 22°C. After centrifugation at 11,000 xg, the supernatant was analyzed by SDS-PAGE. The proteins were silver-stained.

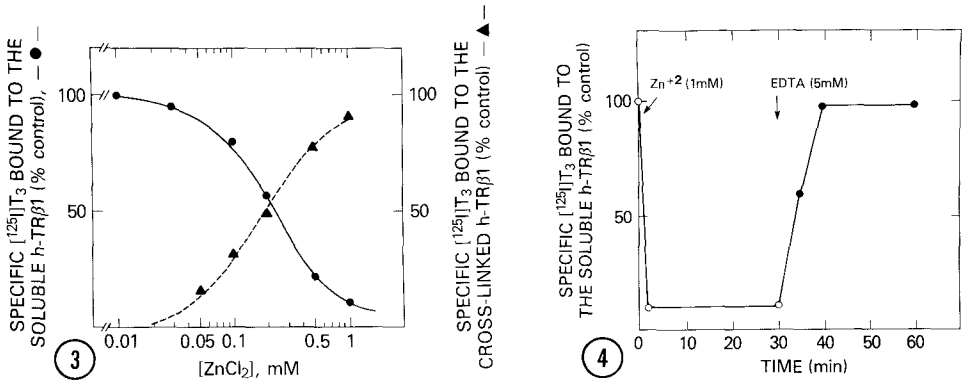

Figure 3. Binding of $[^{125}I]T_3$ to soluble and cross-linked h-TRβ1.

h-TRβ1 (0.4 μg/0.5 mL) in buffer B was incubated with 0.2 nM $[^{125}I]T_3$ and $ZnCl_2$ as described in Figure 1. Soluble (●) and cross-linked h-TRβ1 (▲) were determined as described in Methods.

Figure 4. Time-dependent reversal of the Zn^{+2}-induced cross-linking of h-TRβ1.

h-TRβ1 (0.2 μg/0.25 mL) in buffer B was incubated with 0.2 nM $[^{125}I]T_3$ for 1 hr at 22°C. $ZnCl_2$ (1 mM) was added. After 30 minutes, 5 mM EDTA was added and at the designated time, soluble h-TRβ1-bound $[^{125}I]T_3$ was determined.

reducing the soluble form of h-TRβ1 with an apparent half-maximal concentration of 0.02, 0.03, and 0.045 mM, respectively. Cu^{+2} is weaker than Zn^{+2} with a half maximal concentration of 0.3 mM. Mn^{+2} is the least effective with an apparent half-maximal concentration greater than 1 mM. In contrast, Ca^{+2} and Mg^{+2} have no effect (data not shown). We also analyzed $[^{125}I]T_3$ bound to the soluble form of h-TRβ1 in the presence of increasing concentrations of $ZnCl_2$ by using a Dowex 1-X8 ion exchange method. Similar results were obtained.

To understand whether complexing Zn^{+2} with h-TRβ1 resulted in the formation of aggregates, we separated the soluble form and the cross-linked h-TRβ1 in the binding mixture by centrifugation. The amounts of the soluble h-TRβ1 which remained in the supernatant was determined by SDS-PAGE. Figure 2 shows that the soluble h-TRβ1 detected by silver staining was reduced by Zn^{+2} in a concentration-dependent manner with the apparent half-maximal concentration of ~200 μM. These results are consistent with the findings shown in Figure 1. The pellet was counted to determine the $[^{125}I]T_3$ bound to the aggregated h-TRβ1. As shown in Figure 3, the $[^{125}I]T_3$ bound to the aggregated h-TRβ1 increased concomitantly with the increasing Zn^{+2} concentration. Similar results were also obtained when h-TRβ1 was preincubated with Zn^{+2} first followed by $[^{125}I]T_3$ binding. These results indicated Zn^{+2} induced cross-linking of h-TRβ1 to form aggregates. Furthermore, the cross-linking of h-TRβ1 had no effect on the specific hormone binding activity. Identical results were obtained for Ni^{+2} and Co^{+2} (data not shown).

To determine whether the cross-linking of h-TRβ1 induced by Zn^{+2} is a reversible process, we first added 1 mM of $ZnCl_2$ to induce aggregation. Approximately 90% of T_3 binding activity was lost in the soluble fraction of h-TRβ1. EDTA (5 mM) was added and the restoration of T_3 binding was monitored in a time-dependent manner. Figure 4 shows that at 22°C, 60% of the initial activity was restored after 5 min and after 10 min, nearly 100% of the activity was restored. The recovered h-TRβ1 is also shown by SDS-PAGE (Figure 2, lane 6). However, addition of DTT (10 mM) only recovered ~70% of the T_3 binding activity (data not shown). These results suggested that cross-linking involved amino acid residues other than cysteine.

The present study showed that complexing Zn^{+2} with h-TRβ1 resulted in cross-linking of h-TRβ1. The cross-linking of h-TRβ1 is reversible, however, by the addition of EDTA or DTT. It is of importance to note that the cross-linking of h-TRβ1 induced by Zn^{+2} had no effect on hormone binding, suggesting that the cysteines and/or histidines involved are not essential for T_3 binding. The cross-linking is not an artifact due to the purification of h-TRβ1 from *E. coli*, as the soluble form of h-TRβ1 in *E. coli* lysate (7) also complexed with Zn^{+2} similarly (data not shown). Futhermore, it is not due to the concentration of receptor (0.4 - 0.8 μg/ml) used in the present study. We evaluated Zn^{+2}-induced aggregation of h-TRβ1 in the concentration range of 800 - 0.4 ng/mL. We found that aggregation of h-TRβ1 occurred as low as 0.4 ng/ml in the presence of 0.2 mM of $ZnCl_2$ (data not shown).

The present results differed from those described by Surks *et al.* (5) and Lu *et al.* (6). The reasons are unclear. However, in evaluating the Zn^{+2} effect on hormone binding to c-*erb*A receptor family, the method used to separate the free and receptor-bound hormone has to be carefully chosen. Methods which retain the aggregates together with the absorbed-unbound hormone (G-25 Sephadex column) or pellet the aggregates together with the absorbed-unbound hormone (ion exchange resin or charcoal method) will only yield ligand-bound soluble form of the receptor. This might lead to an erroneous interpretation.

REFERENCES

1. DeGroot, L. J., Nakai, A., Sakurai, A., and Macchia, E. (1989) J. Endocrinol. Invest. 12, 843-861.
2. Samuels, H. H., Forman,B. M., Horowitz, Z. D., and Ye, Z. S. (1988) J. Clin. Invest. 81, 957-967.
3. Hard, T., Kellenbach, E., Boelens, R., Maler, B. A., Dahlman, K., Freedman, L. P., Carlstedt-Duke, J., Yamamoto, K. R., Gustafsson, J., and Kaptein, R. (1990) Science 249, 157-160.
4. Freedman, L.P., Luisi, B. F., Korszun, Z. R., Basavappa, R., Sigler, P. B., and Yamamoto, K. R. (1988) Nature, 543-546.

5. Surks, M. I., Ramirez, I. J., SHapiro, L. E., and Kumara-Siri, M. (1989) J. Biol. Chem. 264, 9820-9826.
6. Lu, C. L., Chan, J. Y. C., and Walfish, P. G. (1990) Biochem. Int. 21, 191-198.
7. Lin, K. H., Fukuda, T., and Cheng, S. Y. (1990) J. Biol. Chem. 265, 5161-5165.
8. Berg, J. M. (1986) Science 232, 485-487.
9. Samuels, H. H., Tsai, J. S., Casanova, J., and Stanley, F. (1974) J. Clin. Invest. 54, 853-865.
10. Kitagawa, S., Obata, T., Hasumura, S., Pastan, I., and Cheng, S.-Y. (1987) J. Biol. Chem. 262, 3903-3908.
11. Brtko, J., Knapp, J., DeGroot, L. J. (1987) Endocrinol. Exp. 21, 251-256.