FLUORESCENT RHODAMINE-LABELED THYROID HORMONE DERIVATIVES

Synthesis and binding to the thyroid hormone nuclear receptor

Sheue-yann CHENG, Norman L. EBERHARDT*, Jacob ROBBINS, John D. BAXTER* and Ira PASTAN+

Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, +Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014 and **Endocrine Research Division, Department of Medicine, Biochemistry and Biophysics, and Metabolic Research Unit, University of California, San Francisco, CA 94143, USA*

Received 17 January 1979

1. Introduction

Studies of the transport, intracellular distribution and interaction of thyroid hormones with specific subcellular components have depended upon the use of radiolabeled hormones. Application of this methodology to the study of transport in intact cells, however, is limited because of the difficulties in differentiating transported hormone from bound hormone at the cell surface. Therefore, it is important to devise alternate techniques for studying the transport of thyroid hormones in intact cells. A highly sensitive technique termed 'video intensification microscopy' has been used [1–3] to study the binding and internalization of fluorescence-labeled peptide hormones and proteins in living cells. To apply this technique to thyroid hormones we have synthesized rhodamine-labeled derivations of iodothyronines directly to visualize their uptake, transport and distribution in cells.

To determine if the derivatives retained biological specificity, we studied their interaction with the thyroid hormone nuclear receptor [7]. A growing body of evidence indicates that many responses of thyroid hormones may be explained by their interaction with a chromatin-localized receptor which subsequently influences the expression of specific genes (reviewed [4–6]). Studies from a number of laboratories indicate that the receptor binds T_3, T_4 and other thyroid hormone analogues in proportion to their biological activity [7–10].

Abbreviations: T_3, 3,5,3'-tri-iodo-L-thyronine; T_4, 3,5,3',5'-tetra-iodo-L-thyronine; T_0, L-thyronine; [^{14}C]T_0, 4-hydroxy-phenoxy-[U-^{14}C]tyrosine; TRITC, tetramethyl rhodamine isothiocyanate; TLC, thin-layer chromatography

2. Materials and methods

[^{125}I]T_3 and [^{125}I]T_4, with spec. act. 1300 μCi/μg and 1000 μCi/μg, respectively, were obtained from New England Nuclear. Non-radioactive T_4 and T_3 were purchased from CalBiochem. T_0 was kindly provided by Dr Paul Block, River Research, 4059 River Road, Toledo, OH 43614. TRITC (mixed isomers) was obtained from Research Organic, Cleveland, OH. Preparative and analytical thin-layer plates were from Whatman, New Jersey. Solvent systems for TLC were: (A) upper phase of ethyl acetate:methanol:water (5:2:3, v/v/v) which was adjusted to pH 10.5 with the addition of triethylamine; (B) upper phase of ethyl acetate:methanol:2 N NH_4OH (5:2:3, v/v/v); (C) hexane:*tert*-amyl alcohol:methanol:water (1:6:2:1; v/v/v/v). Absorption spectra were measured in a Cary 14 spectrophotometer.

2.1. *Synthesis of [^{125}I]T_3- and [^{125}I]T_4-rhodamine*

The synthetic scheme for [^{125}I]T_3-rhodamine is illustrated in fig.1. In a typical synthesis, 3 μmol each of TRITC and [^{125}I]T_3 (0.5 Ci/mol) in 0.1 ml pyridine:water:triethylamine (9:1.5:0.1, v/v/v) were reacted for 1 h at room temperature. Purification was carried out by repeated preparative TLC in solvent

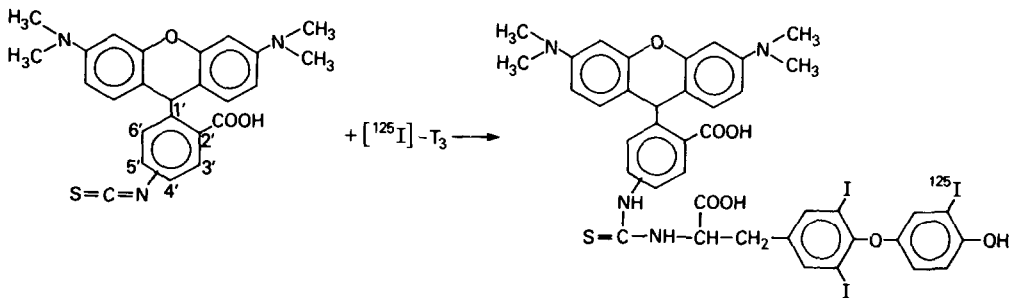

Fig.1. Synthetic scheme for the preparation of [^{125}I]T_3-rhodamine.

system A. The purple radiolabeled bands with R_F ~0.25 and ~0.27, designated as T_3-rhodamine-I and T_3-rhodamine-II, respectively, were eluted with methanol. After evaporation of methanol, the dry residues were stored in the dark at −20°F and were stable for periods of at least 6 months. The radiolabeled derivatives were used as markers for purification. The specific activity was maintained sufficiently low so that subsequent nuclear receptor binding assays utilizing [^{125}I]T_3 were not affected.

[^{125}I]T_4-rhodamine was synthesized and purified similarly except that the radiolabeled bands eluted had R_F ~0.13 and ~0.15 were designated as [^{125}I]T_4-rhodamine-I and [^{125}I]T_4-rhodamine-II, respectively.

2.2. *Synthesis of [^{14}C]T_0-rhodamine*

[^{14}C]T_0 was synthesized by coupling of 3,5-diiodo-[U-^{14}C]tyrosine with 4-hydroxy-3,5-di-iodophenylpyruvic acid to form ^{14}C-labeled T_4 [11] followed by subsequent de-iodination. ^{14}C-Labeled T_4 was first de-iodinated with a mixture of hypophosphorous acid and hydriodic acid to form ^{14}C-labeled 3,5-di-iodothyronine [12] which was further de-iodinated to [^{14}C]T_0 by catalytic hydrogenation in the presence of 5% Pd on alumina.

The method used to covalently link T_0 to rhodamine was the same as described for the synthesis of T_3- or T_4-rhodamine. However, solvent system B was used in preparative TLC and the eluted band had R_F ~0.15. The unresolved 4′ and 5′ isomeric mixture of T_0-rhodamine was used in the nuclear receptor binding assays.

2.3. *Binding to thyroid hormone nuclear receptors*

The binding assays were carried out as detailed in [7].

3. Results and discussion

The thyroid hormones, T_3 and T_4, and the biologically inactive T_0 were attached through their amino groups to tetramethyl rhodamine via a thiourea linkage (fig.1). Since the starting material, TRITC, was commercially obtained as a mixture of 4′ and 5′ isomers, two isomeric derivatives of T_3 would be expected. Two radiolabeled rhodamine-containing derivatives with similar chromatographic mobilities were observed during purification and were subsequently isolated. The slow- and fast-migrating derivatives, T_3-rhodamine-I and T_3-rhodamine-II (see section 2), respectively, presumably represent the attachment of T_3 to the 4′ or 5′ position of rhodamine. The formation of the T_3-rhodamine conjugate was established by:

(i) Its chromatographic mobility which is lower than either of the initial reactants;
(ii) The presence of T_3 as determined by incorporation of ^{125}I radioactivity,
(iii) Its characteristic absorption spectrum which is consistent with the presence of rhodamine and T_3 moieties. Purity was established by TLC on silica gel and cellulose plates in solvent system A and C. The absence of contamination by free hormone was confirmed by autoradiography and counting segments of the thin-layer chromato-

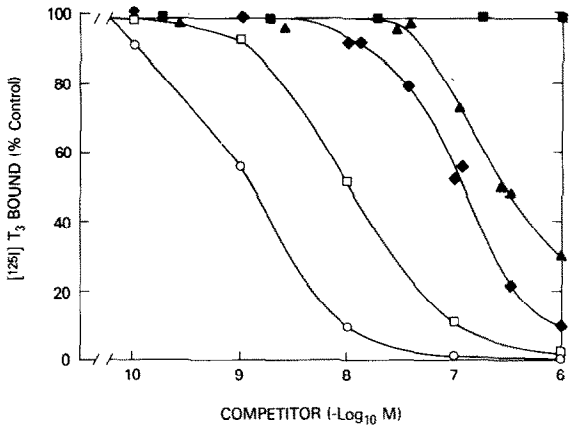

Fig.2. Competition of $[^{125}I]T_3$ binding to the solubilized thyroid hormone nuclear receptor by unlabeled T_3 (circles), T_4 (open squares), T_3-rhodamine-II (solid diamonds), T_4-rhodamine-II (solid triangles) and T_0-rhodamine (solid squares). The nuclear receptor [7] was solubilized from purified rat liver nuclei and was incubated with $[^{125}I]T_3$ at 1 nM final conc. with varying concentrations of the indicated competitors for 2 h at 22°C. Bound hormone was separated from free hormone by passage of the incubation mixtures over 2.0 ml Sephadex G-25 columns and subsequent determination of bound radioactivity. The individual data points represent the average of duplicate determinations.

grams. The purity of $[^{125}I]T_4$- and $[^{14}C]T_0$-rhodamine was established similarly.

Figure 2 shows the results of the competition of $[^{125}I]T_3$ binding to the receptor by unlabeled T_3, T_4, T_3-rhodamine-II, T_4-rhodamine-II and T_0-rhodamine. These data indicate that T_3- and T_4-rhodamine-II both bind to the receptor with affinities ~2 orders of magnitude lower than T_3. The calculated [7] K_d values for the binding of T_3-rhodamine-II and T_4-rhodamine-II to the receptor are 20.3 nM and 70.8 nM, respectively. Thus, the derivatives bind to the receptor at concentrations which can be reasonably achieved in intact cells. The data also indicate that T_3-rhodamine-II binds to the receptor more avidly than T_4-rhodamine-II, although the difference in the affinities of the two derivatives is somewhat smaller than with the parent hormones (fig.2) [7–10]. In addition, T_0-rhodamine does not interact with the receptor over the concentration range employed in these studies. Since T_0 is biologically inactive, we conclude that the binding of the thyroid hormone-rhodamine derivatives to the receptor results from biospecific interactions.

The slow- and fast-migrating T_3- and T_4-rhodamine derivatives possess slightly different affinities for the receptor (table 1). The fast-migrating derivatives have higher affinities than the slow-migrating derivatives. Although the exact linkage position for each of the derivatives is unknown, the data indicate that the orientation of rhodamine with respect to the hormone has a minor influence on the binding affinities of the derivatives to the receptor.

The synthesis of fluorescein-labeled T_4 has been reported [13]; however, its physico-chemical properties and biological specificity have not been examined. Furthermore, fluorescein has a relatively faster fluorescence bleaching rate than rhodamine thereby decreasing the sensitivity of its detection by 'video intensification microscopy'.

Our data indicate that the rhodamine-labeled derivatives of thyroid hormones interact with nuclear thyroid hormone receptors in a manner which reflects the binding specificities of the parent hormones. These data therefore indicate that these derivatives may be useful in studies of thyroid hormone interaction with intact cells.

Table 1
Apparent equilibrium dissociation constants for the binding of rhodamine-labeled thyroid hormones to the nuclear receptor

Rhodamine-labeled thyroid hormones	K_d (nm)[a]
T_3-rhodamine-II	20.3
T_3-rhodamine-I	38.3
T_4-rhodamine-II	70.8
T_4-rhodamine-I	203

[a] The dissociation constants were calculated from the equation:

$$K_d = C_{50} \cdot K_T/(T + K_T)$$

where K_d = apparent dissociation constant of the competitor, C_{50} = concentration of competitor required to produce 50% inhibition, K_T = dissociation constant of T_3, and T = concentrations of $[^{125}I]T_3$ utilized in the assay. The receptor preparation utilized in these studies had a T_3 K_d of 0.221 nM as determined by Scatchard analysis

References

[1] Willingham, M. C. and Pastan, I. (1978) Cell 13, 501–507.
[2] Schlessinger, J., Shechter, Y., Willingham, M. C. and Pastan, I. (1978) Proc. Natl. Acad. Sci. USA 75, 2659–2663.
[3] Maxfield, F. R., Schlessinger, J., Shechter, Y., Pastan, I. and Willingham, M. C. (1978) Cell 14, 805–810.
[4] Oppenheimer, J. H., Schwartz, H. L., Surks, M. I., Koerner, D. and Dillman, W. H. (1976) Rec. Prog. Hor. Res. 32, 529–565.
[5] Surks, M. I. and Oppenheimer, J. H. (1976) in: Hormone–Receptor Interaction. (Levy, G. S. ed) pp. 373–384, Marcel Dekker, New York.
[6] Latham, K. R., MacLeod, K. M., Papavasiliou, S. S., Martial, J. A., Seeburg, P. H., Goodman, H. M. and Baxter, J. D. (1979) in: Hormone Receptors. (Goodman, H. M. and Baxter, J. D. eds) Academic Press, New York, in press.
[7] Latham, K. R., Ring, J. C. and Baxter, J. D. (1976) J. Biol. Chem. 251, 7388–7397.
[8] Koerner, D., Schwartz, H. L., Surks, M. I. and Oppenheimer, J. H. (1975) J. Biol. Chem. 250, 6417–6423.
[9] Jorgensen, E. C., Murry, W. J. and Block, P., jr (1974) J. Med. Chem. 17, 434–439.
[10] Samuels, H. H. and Tsai, J. S. (1973) Proc. Natl. Acad. Sci. USA 70, 3488–3492.
[11] Shiba, T. and Cahnmann, H. J. (1962) J. Org. Chem. 27, 1773–1778.
[12] Sorimachi, K. and Cahnmann, H. J. (1977) Endocrinol. 4, 1276–1280.
[13] Smith, D. S. (1977) FEBS Lett. 77, 25–27.