Identification of Thyroid Hormone Receptors in Rat Liver Nuclei by Photoaffinity Labeling with L-Thyroxine and Triiodo-L-thyronine

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ABSTRACT: Photoaffinity labeling of rat liver nuclear extract with underivatized thyroid hormones was performed after incubation with 1 nM [3′,5′-^{125}I]thyroxine ([^{125}I]T_4) or [3′-^{125}I]triiodothyronine ([^{125}I]T_3) by irradiation with light above 300 nm. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the covalently photolabeled nuclear extract revealed four distinct hormone binding proteins of molecular masses 96, 56, 45, and 35 kilodaltons (kDa), respectively. Distribution of the hormone among these proteins was similar for T_4 and T_3. The 56- and 45-kDa proteins were the most prominently labeled. The specificity of the photoattachment of thyroid hormones to these nuclear proteins was verified by the irradiation of eight randomly chosen proteins and two proteins known to have thyroid hormone binding sites, human thyroxine binding globulin and bovine serum albumin. Only the latter two were photolabeled with [^{125}I]T_4. Competition studies performed by incubating nuclear extracts with [^{125}I]T_4 or [^{125}I]T_3 in the presence of increasing amounts of the corresponding unlabeled hormone (10-, 100-, and 1000-fold molar excess) demonstrated that (1) photoattachment of labeled T_3 or T_4 to the 56- and 45-kDa proteins was inhibited by 67–78% and 73–85%, respectively, after incubation with a 1000-fold molar excess of unlabeled hormone, (2) in the presence of lower molar excesses of the corresponding competitor (10- and 100-fold), photoattachment of labeled T_3 or T_4 to the 56- and 45-kDa receptors was gradually inhibited to a similar extent on both proteins, and (3) the 35- and 96-kDa proteins, although having thyroid hormone binding sites, display lower binding activities since the inhibition of photoattachment of labeled T_3 or T_4 by a 1000-fold molar excess of unlabeled hormone did not exceed 30–42% and 26–49%, respectively. Hence, only the 56- and 45-kDa proteins are thyroid hormone receptors. The similar behavior of the 56- and 45-kDa receptors in the competition studies suggests a structural relationship between the two proteins. Digestion of the 56- and 45-kDa proteins with *Staphylococcus aureus* V8 protease yielded virtually identical peptide patterns, indicating that both receptor proteins share homologous amino acid sequences.

Certain biological actions of the thyroid hormones 3,5,3′-triiodo-L-thyronine (T_3)[1] and T_4 are believed to be mediated by the binding of the hormone to chromatin-linked receptors (Oppenheimer, 1975; Baxter et al., 1979; Samuels, 1983). Molecular masses from 48 to 70 kDa have been determined for the receptor in rat liver nuclei by gel filtration of nuclear extracts (Surks et al., 1973; DeGroot et al., 1974; Latham et al., 1976; Torresani et al., 1978) or sucrose gradient centrifugation (Jump et al., 1981). By the use of ionizing irradiation, the "target size" of the functional unit involved in the binding of T_3 was found to be 59 kDa (Gruol & Kempner, 1982). Covalent binding of the *N*-bromoacetyl derivatives of the thyroid hormones, followed by high-performance liquid chromatography and SDS–polyacrylamide gel electrophoresis, revealed a nuclear binding protein of 56 kDa (Nikodem et al., 1980). More recently, we introduced a method of photoaffinity labeling with the underivatized hormones (van der Walt et al., 1982). By use of this new method with either nuclear extracts or whole nuclei, thyroid hormone binding proteins of distinct molecular masses were detected. Among these, the 56- and 45-kDa proteins were the most prominent ones. They are of particular interest since they fall within the molecular size range reported by the above-mentioned investigators and by Pascual et al. (1982) and David-Inouye et al. (1982) for the nuclear thyroid hormone receptor.

In the present study, we used photoaffinity labeling with the underivatized thyroid hormones followed by SDS–polyacrylamide gel electrophoresis to (1) separate the major nuclear hormone binding proteins, (2) determine whether or not the covalent photoattachment of thyroid hormones to these proteins is specific, (3) probe the distribution of high- and low-affinity sites among the different binding proteins (competition studies), and (4) investigate a possible structural relationship between the two major high-affinity binding proteins (56 and 45 kDa).

Materials and Methods

Reagents. Carrier-free [3′-^{125}I]T_3 (3300 μCi/μg) and [3′,5′-^{125}I]T_4 (5500 μCi/μg) were purchased from New England Nuclear. T_4 was obtained from Glaxo Laboratories and then further purified according to Kerwin (1972). T_3 (free acid) was from Calbiochem and *Staphylococcus aureus* V8 protease from Miles Laboratories. Highly purified human thyroxine binding globulin was a gift from Dr. J. S. Marshall, Case Western Reserve University School of Medicine. Other proteins were from various commercial sources.

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[1] Abbreviations: T_3, 3,5,3′-triiodo-L-thyronine; T_4, L-thyroxine; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Table I: Competition of Labeled Hormone with Increasing Amounts of Unlabeled Hormone[a]

hormone binding protein		1 nM [^{125}I]T_4 + T_4 molar excess of T_4				1 nM [^{125}I]T_3 + T_3 molar excess of T_3				1 nM [^{125}I]T_3 + T_4 molar excess of T_4			
		0	10	100	1000	0	10	100	1000	0	10	100	1000
96 kDa	cpm[b]	4330	3850	2650	2170	1020	890	800	590	820	740	670	530
	%[c]	0	11	39	49	0	12	21	42	0	10	18	34
56 kDa	cpm	10720	7760	5370	2350	2560	2000	1300	940	2030	1780	1400	1190
	%	0	28	50	78	0	22	49	67	0	12	31	42
45 kDa	cpm	9000	5620	4400	1280	2220	1570	1270	740	2480	2040	1640	1390
	%	0	37	51	85	0	29	43	73	0	18	34	44
35 kDa	cpm	5220	5070	4850	3850	810	710	670	570	900	880	810	700
	%	0	3	7	26	0	12	17	30	0	3	11	22

[a] The same rat liver nuclear extract (100 μg of protein) was incubated with (1) 1 nM [^{125}I]T_4 in the absence or presence of 10-, 100-, or 1000-fold molar excesses of T_4, (2) 1 nM [^{125}I]T_3 in the absence or presence of the same molar excesses of T_3, or (3) 1 nM [^{125}I]T_3 in the absence or presence of the same molar excesses of T_4. Incubation was followed by irradiation and electrophoresis as described in the legent to Figure 1. [b] Radioactivity of the 96-, 56-, 45-, or 35-kDa peak materials. [c] Inhibition of photoattachment of labeled hormone is expressed as a percentage of the radioactivity measured in the absence of unlabeled competitor. Percentages are not corrected for nonspecific binding. If a correction is applied by subtracting the amount of radioactivity which becomes covalently incorporated in the presence of a 1000-fold excess of unlabeled competitor, these percentages become slightly higher. Thus, the percentages for the 56- and 45-kDa proteins shown for 1 nM [^{125}I]T_4 + T_4 data rise from 28% and 37% to 35% and 44%, respectively, when a 10-fold excess of competitor is used and from 50% and 51% to 64% and 59%, respectively, when a 100-fold excess of competitor is used. This means that correction for nonspecific binding results in percent inhibition values which are 7–14% higher than those calculated without correction. For reproducibility of photoaffinity labeling, see Table I of the following paper (Dozin et al., 1985).

Preparation of Nuclear Extract. All steps were carried out at 0–4 °C. Nuclei from rat liver were prepared according to Blobel & Potter (1966) except that all solutions contained 1 mM phenylmethanesulfonyl fluoride, added just before use from a 100 mM solution in ethanol. Nuclear pellets were washed once with 20 mM Tris-HCl, pH 7.6, containing 0.32 M sucrose, 4 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% Triton X-100 and once with the same buffer without Triton. These washes were done either before or after passage of the nuclei through a cushion of 2.2 M sucrose containing 2 mM $MgCl_2$, without affecting the recovery of nuclear proteins (SW27 rotor, 109000*g*, 75 min). The nuclear pellets were extracted for 60 min with 20 mM Tris-HCl, pH 7.85, containing 0.4 M NaCl, 2 mM EDTA, 2 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride (extraction buffer). The extract was then centrifuged for 30 min at 109000*g*. In some cases, pellets were kept at −70 °C (no longer than 1 month) and extracted before experiments.

Photoaffinity Labeling. Nuclear extracts (250 μL, 80–120 μg of protein) were incubated with 1 nM [^{125}I]T_4 or [^{125}I]T_3 alone or in the presence of different molar excesses of unlabeled competitor (T_4 or T_3) at 2 °C for 16–18 h to allow equilibrium to be reached. The samples were then irradiated (450-W Canrad-Hanovia mercury arc lamp 679A-36) at 2 °C for 30 min with light in the visible and near-UV regions. Virtually all high-energy UV radiation (below ~300 nm) had been filtered out by an immersion well of borosilicate glass (van der Walt & Cahnmann, 1982). This well permitted light transmission of 1, 50, and 89% at 294, 323, and 360 nm, respectively. Labeling of thyroxine binding globulin and other highly purified proteins with [^{125}I]T_4 was performed under the same conditions. In a few experiments, a more efficient source for light above 300 nm was used (Osram HBO/2 lamp), in which case irradiation times varied from 20 to 80 s.

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide slab gel electrophoresis was performed according to Laemmli (1970). Acrylamide and *N,N'*-methylenebis(acrylamide) concentrations were 10% and 0.24%, respectively, unless noted otherwise. The photolabeled samples (200–250 μL) were boiled for 5 min in the presence of 1% SDS and 5% 2-mercaptoethanol and then electrophoresed until bromophenol blue reached the bottom of the gel. The gels were fixed in 10% trichloroacetic acid, stained with Coomassie blue, and washed, first with acetic acid/methanol/water (1:2:7) for 12 h and then repeatedly with acetic acid (7%) for an additional 12 h to remove noncovalently bound hormone which would otherwise be detected as a sharp peak of radioactivity at the bottom of the gel. The gel strips were cut in 2-mm slices, and the radioactivity in each slice was measured in a Beckman γ 9000 spectrometer. Control samples which were not irradiated were analyzed in an identical manner. No significant amount of radioactivity was detected in any gel slice.

Enzymatic Digestion of [^{125}I]T_4-Photolabeled Nuclear Proteins. Enzymatic digestion with *Staphylococcus aureus* V8 protease was performed according to Cleveland et al. (1977). A [^{125}I]T_4-photolabeled nuclear extract was electrophoresed on a 10% SDS–polyacrylamide gel; slices containing the labeled proteins of 56 and 45 kDa were placed in separate slots of a 15% SDS–polyacrylamide slab gel and overlaid with a solution of 2.5 μg of *Staphylococcus aureus* V8 protease in 15 μL of sample buffer (Laemmli, 1970) and then with 15 μL of 10% glycerol. The samples were electrophoresed at room temperature ~2 cm deep into the 3-cm stacking gel. The power was turned off for 30 min to allow proteolysis to take place, and then electrophoresis was resumed. The gel was stained and destained and, after drying, exposed for 2 days to a Kodak XAR2 film, using an intensifying screen. The autoradiogram was scanned with a densitometer.

Results

Photoaffinity Labeling of Rat Liver Nuclear Extract. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of over 100 nuclear extracts photolabeled with 1 nM [^{125}I]T_4 or [^{125}I]T_3 gave radioactivity profiles with peaks corresponding to proteins of molecular masses of 96, 56, 45, and 35 kDa, respectively. The ratios of the peak areas, as determined by the total radioactivity under each peak, were in the same order, 0.28:1.0:0.90:0.36 (mean values of 18 typical experiments), regardless of whether labeled T_4 or T_3 was used. Representative profiles obtained with either tracer are shown in the left column of Figure 1. Virtually identical radioactivity patterns were observed when using either the Canrad-Hanovia mercury lamp or the Osram HBO/2 setup (not shown).

A comparison of the amount of covalently incorporated radioactivity obtained with [^{125}I]T_4 and with [^{125}I]T_3 shows that the former resulted in a roughly 5 times higher radioincorporation than the latter (Table I). This was to be expected since (1) the photoattachment efficiency of [3′,5′-^{125}I]T_4 has been shown to be considerably higher than that of [3′-^{125}I]T_3, which is a consequence of the fact that the photolytic fission

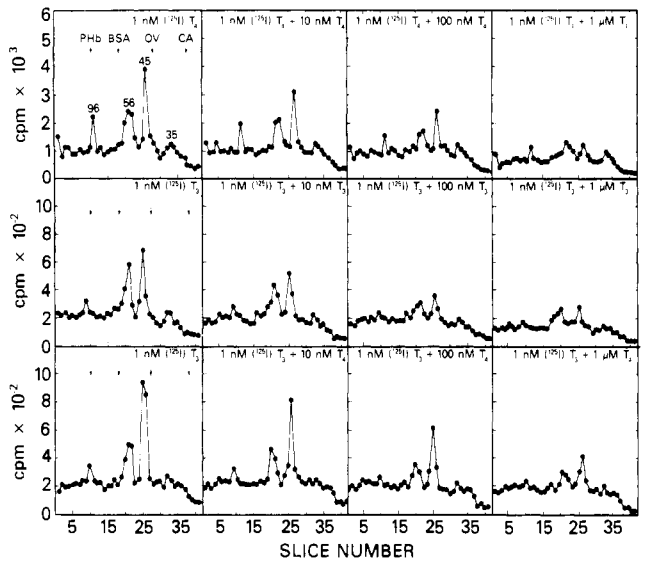

FIGURE 1: SDS–polyacrylamide gel electrophoresis of rat liver nuclear extract after photoaffinity labeling. Rat liver nuclear extract (100 μg of protein) was incubated with 1 nM [^{125}I]T_4 in the absence or presence of a 10-, 100-, or 1000-fold molar excess of unlabeled T_4 (top row), with 1 nM [^{125}I]T_3 in the absence or presence of the same molar excesses of unlabeled T_3 (middle row), or with 1 nM [^{125}I]T_3 in the absence or presence of the same molar excesses of unlabeled T_4 (bottom row). The samples were irradiated (30 min) with light above 300 nm and electrophoresed on a 10% SDS–polyacrylamide gel. After the gels were stained and destained, they were cut in 2-mm slices, and the radioactivity of each slice was counted. Marker proteins are phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).

of the hormones into phenyl radicals (incorporated into the binding site of the protein) and iodine radicals (mainly reduced to iodide) occurs predominantly, but not exclusively, in the outer ring of the molecule [see discussion in van der Walt et al. (1982)], and (2) thiols which are known to enhance the binding of T_3 through an increase in the affinity of the binding sites for the hormone (Torresani & DeGroot, 1975; Dozin et al., 1981) were omitted in our photoattachment experiments because they are powerful scavengers of the free radicals created by irradiation and therefore inhibit drastically the covalent photoincorporation of radioactivity in proteins (unpublished observations). Photoincorporation efficiency in the absence of thiols was ~0.5% for [3′-^{125}I]T_3 and ~2% for [3′,5′-^{125}I]T_4. Radioactivity bound to the receptor(s) prior to irradiation was determined by the resin uptake method of Torresani & DeGroot (1975).

Specificity of Photoaffinity Labeling. The specificity of photoaffinity labeling has been established by photoaffinity labeling a mixture of nine proteins which were selected either with respect to their molecular weights or because they are known to have thyroid hormone binding properties. A solution containing a mixture of carbonic anhydrase, alkaline phosphatase, ovalbumin, catalase, bovine serum albumin, conalbumin, phosphorylase *b*, β-galactosidase, and histone H_1 was incubated in the presence of 1 nM [^{125}I]T_4 and analyzed, after irradiation, by SDS–polyacrylamide gel electrophoresis. The resulting radioactivity profile (Figure 2A) shows that significant photoattachment of labeled T_4 took place only with bovine serum albumin. As another control, highly purified human thyroxine binding globulin (7.4 nM) was incubated with an equimolar amount of [^{125}I]T_4 in 0.2 M Tris-HCl, pH 8.6, and then irradiated in an identical manner. The marked peak of radioactivity obtained after SDS gel electrophoresis indicates that T_4 had been efficiently incorporated into the thyroxine binding globulin by covalent photoattachment

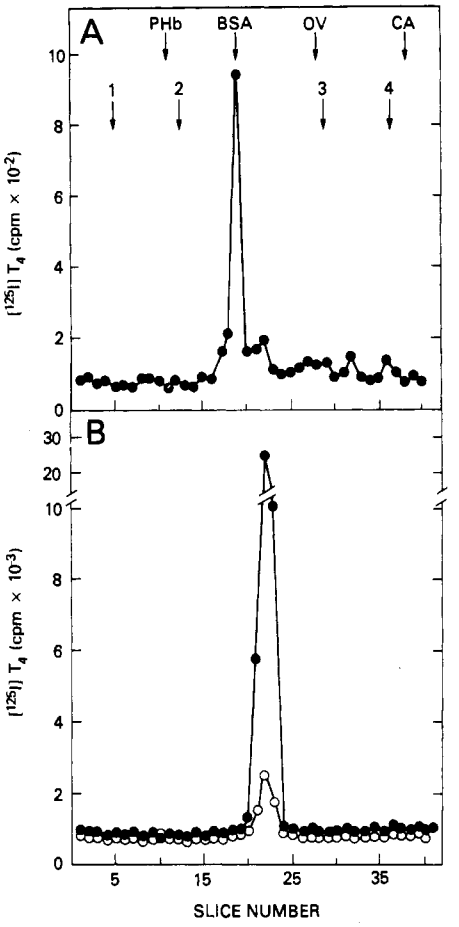

FIGURE 2: SDS–polyacrylamide gel electrophoresis of various proteins photolabeled with [^{125}I]T_4. (A) A solution containing nine purified proteins [β-galactosidase (1), phosphorylase *b* (PHb), conalbumin (2), bovine serum albumin (BSA), catalase (3), alkaline phosphatase (4), ovalbumin (OV), histone H1 (5), and carbonic anhydrase (CA) (1 or 5 μg of each protein in a total volume of 200 μL of extraction buffer)] was incubated with 1 nM [^{125}I]T_4. (B) Highly purified human thyroxine binding globulin (0.1 μg in 250 μL of 0.2 M Tris-HCl, pH 8.6) was incubated with 7.4 nM [^{125}I]T_4 in the absence (●) or in the presence (○) of 7.4 μM unlabeled T_4. Both solutions (A and B) were irradiated and worked up as described in the legend to Figure 1. The radioactivity recovered under the smaller peak (○) of panel B represents 18% of that under the larger peak (●).

(Figure 2B, closed circles). It has been established in additional experiments (not shown) that the limit of detectability of thyroxine binding globulin is less than 10^{-10} M.

Since only the two proteins with known specific binding sites for T_4 whose K_a is greater than 10^5 M^{-1}, viz., bovine serum albumin ($K_a = 2.0 \times 10^6$ M^{-1}) (Steiner et al., 1966) and human thyroxine binding globulin ($K_a = 1.5 \times 10^{10}$ M^{-1}) (Robbins & Rall, 1979), gave peaks of covalently bound radioactivity, while the eight other proteins did not, it is reasonable to assume that the four covalently labeled nuclear proteins described above owe their radioactivity to specific labeling.

Competition Studies. To obtain further information about the distribution of high- and low-affinity sites among the four thyroid hormone binding nuclear proteins, competition studies were performed. Rat liver nuclear extract was incubated with (1) 1 nM [^{125}I]T_4 in the absence or presence of 10-, 100-, and 1000-fold excess of unlabeled T_4, (2) 1 nM [^{125}I]T_3 in the absence or presence of 10-, 100-, and 1000-fold excess of unlabeled T_3, or (3) 1 nM [^{125}I]T_3 in the absence or presence of 10-, 100-, and 1000-fold molar excess of unlabeled T_4.

The inhibition of covalent incorporation of radioactivity in the 96-, 56-, 45-, and 35-kDa proteins caused by incubation

with increasing amounts of unlabeled competitor is evidenced by changes in the various peak sizes in Figure 1. They are presented in tabulated form in Table I.

Addition of a 10-fold excess of unlabeled hormone led to an inhibition of covalent incorporation of T_4 radioactivity in the 56- and 45-kDa proteins of 28% and 37%, respectively, and to an inhibition of T_3 radioactivity of 22% and 29%, respectively. Addition of a 100-fold excess increased these percentages to 50% and 51%, respectively, for T_4 and to 49% and 43%, respectively, for T_3.

The same excesses of unlabeled hormone (10- and 100-fold) resulted in only a minimal inhibition of covalent incorporation of radioactivity in the 35-kDa protein. Thus, the 56- and 45-kDa proteins are thyroid hormone nuclear receptors by classical definition (high affinity–low capacity), and the 35-kDa protein, although being a hormone binding protein, cannot be classified as a receptor. The 96-kDa protein was not always detected. When present, incorporation of radioactivity was low.

Nonspecific photoattachment of [^{125}I]T_4 or [^{125}I]T_3 was estimated by competition with a 1000-fold molar excess of the corresponding unlabeled hormone. This excess inhibited the covalent incorporation of radioactivity in the 56- and 45-kDa proteins by 78% and 85%, respectively, when the ligand was T_4, and by 67% and 73%, respectively, when the ligand was T_3, which corresponds to a nonspecific photoattachment ranging from 15% to 33% of total binding. This level of nonspecific binding was about twice that observed (10–15%) under equilibrium conditions with an ion-exchange resin method (Torresani & DeGroot, 1975) when the same excess of unlabeled competitor was used. A similar increase in nonspecific binding upon irradiation (~15%) has also been observed when a synthetic ^{125}I-labeled photoaffinity probe was used for the characterization of the α_1-adrenergic receptor (Leed-Lundberg et al., 1984).

It can be seen from Table I (1 nM [^{125}I]T_4 + T_4 vs. 1 nM [^{125}I]T_3 + T_3 columns) that T_4 competed with [^{125}I]T_4 for hormone binding sites on the 56- and 45-kDa receptors to the same extent as T_3 competed with [^{125}I]T_3, at all concentrations of unlabeled hormone. Cross-competition experiments (1 nM [^{125}I]T_3 + T_3 vs. 1 nM [^{125}I]T_3 + T_4 columns) show that T_3 competed more efficiently than T_4 for T_3 binding sites on the two receptors. Thus, a 10-fold molar excess of unlabeled T_3 inhibited covalent binding of labeled T_3 to the 56- and 45-kDa proteins to the extent of 22% and 29%, respectively, while the same molar excess of unlabeled T_4 inhibited covalent binding of labeled T_3 to the extent of only 12% and 18%, respectively.

Proteolytic Digestion of the 56- and 45-kDa Proteins. The similar behavior of the 56- and 45-kDa proteins observed in the competition experiments suggested a structural relationship between the two proteins. This possibility was investigated by means of partial proteolytic digestion of both proteins with *Staphylococcus aureus* V8 protease after photoaffinity labeling with [^{125}I]T_4. Figure 3 presents scans of the autoradiograms resulting from SDS gel electrophoreses of the digests. Except for the presence of remaining undigested 56-kDa protein seen in Figure 3A, nearly identical radioactivity profiles were obtained with both proteins.

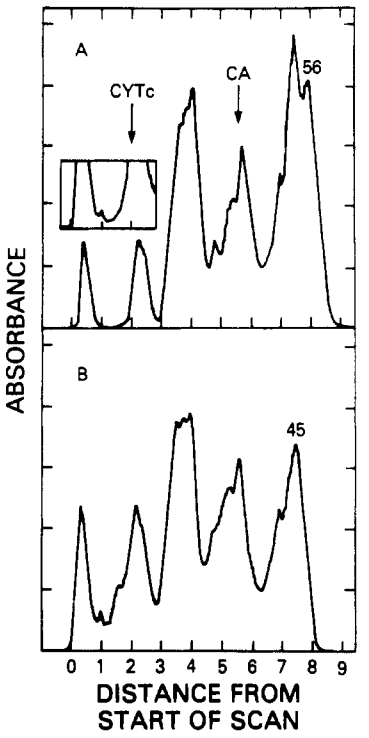

FIGURE 3: Autoradiogram scans of enzymatically digested 56-kDa (A) and 45-kDa (B) nuclear proteins photolabeled with [^{125}I]T. Rat liver nuclear extract was photolabeled with 1 nM [^{125}I]T_4 and resolved on a 10% SDS–polyacrylamide gel (see legend to Figure 1). After being stained and destained, 2-mm gel slices containing the 56- and 45-kDa proteins, respectively, were placed in different slots of a 15% SDS–polyacrylamide gel and overlaid with 2.5 μg of *Staphylococcus aureus* V8 protease in 15 μL of sample buffer (Laemmli, 1970). After a brief electrophoresis, the power was turned off to allow limited proteolysis to take place. Electrophoresis was resumed after 30 min. The gel was stained, destained, dried, and exposed for 2 days to a Kodak XAR2 film. Autoradiograms were scanned with a densitometer. The scan in the inset was obtained by using a 2-fold higher gain. Absorbance and distance are in arbitrary units. Marker proteins are carbonic anhydrase (CA, 30 kDa) and cytochrome *c* (Cyt *c*, 12.5 kDa). Numbers above peaks indicate molecular masses in kilodaltons.

Discussion

Covalent photoattachment of the underivatized hormones is a consequence of C–I bond splitting and therefore takes place at the aromatic ring moiety of the hormone, while covalent attachment mediated by a photolabile group (Pascual et al., 1982; Somack et al., 1982) occurs beyond the amino group of the aliphatic side chain. Although the conformation of binding sites on thyroid hormone receptors is not known, it is likely that the aromatic moiety of the hormone is embedded deeply within the site, while the aliphatic side chain may reach or even extend beyond the confines of the site as has been found to be the case with the bromoacetyl derivatives of pre-albumin (Cheng et al., 1977; Blake et al., 1980) and of thyroxine binding globulin (Siegel et al., 1983). Since C–I bond splitting, although taking place much faster at wavelengths below 300 nm, does not require light in the far-UV region (e.g., at 254 nm) (van der Walt & Cahnmann, 1982), which can be extremely destructive for proteins, we irradiated our samples with light above 300 nm.

Our data show that upon irradiation [^{125}I]T_4 and [^{125}I]T_3 were incorporated in four distinct proteins of molecular masses 96, 56, 45, and 35 kDa, respectively (Figure 1). These four proteins displayed similar relative binding activities for T_3 and T_4 since the distributions of radioactivity among them were comparable when using either one of the tracers, the 56- and 45-kDa proteins carrying the bulk of the covalently incorporated radioactivity.

The degree of nonspecific photoattachment was estimated by preincubation of the extracted nuclear proteins with 1 nM [^{125}I]T_4 or [^{125}I]T_3 in the presence of a 1000-fold molar excess of the corresponding unlabeled competitor (Table I). This excess led to nonspecific binding ranging from 15% to 33%, which was not significantly different from that observed with

highly purified thyroxine binding globulin (18%; Figure 2B). It should be pointed out that, in the absence of thiols, Torresani & DeGroot (1975) observed a 2-fold increase in nonspecific binding of [^{125}I]T_3 to nuclear proteins when a 500–750-fold excess of unlabeled T_3 was used (from 17% to 34% of total binding, as calculated from Figure 2 of these authors). Since thiols have to be omitted in photoaffinity labeling experiments, the increased level of nonspecific binding observed by us should be compared with the level found by Torresani and DeGroot in the absence of thiols.

Competition studies (Table I) with a low molar excess of unlabeled T_3 or T_4 (10- and 100-fold) were performed to assess the distribution of the high- and low-affinity binding sites among the four photolabeled nuclear proteins described above. These amounts of competitor highly inhibited the incorporation of labeled T_3 or T_4 in the 56- and 45-kDa proteins while the degree of inhibition was lower with the 96-kDa protein and only minimal in the case of the 35-kDa protein. Therefore, we conclude that, among the four nuclear binding proteins, the 56- and 45-kDa proteins have high-affinity, low-capacity binding sites and, consequently, should be considered thyroid hormone receptors. The identification of these two nuclear receptors is in agreement with the data obtained by other investigators with derivatives of the thyroid hormones. Pascual et al. (1982) reported two covalently labeled nuclear proteins of 57 and 47 kDa in photolabeled GH_1 cells, but their 47:57-kDa radioactivity ratio was considerably higher than the one observed by us which was about 1:1. David-Inouye et al. (1982) detected only one of these binding proteins (47 kDa) in a rat liver nuclear extract whose prepurification may have led to the loss of the 56-kDa protein. In none of these reports were the 96- and 35-kDa proteins mentioned. The latter cannot be defined as a receptor in view of its lesser binding activity for T_3 and T_4. The nature of the 96-kDa protein which was not present in all nuclear extracts requires further study. This protein could be an altered form of either one of the receptors (e.g., a 56- or 45-kDa protein associated with an inert polypeptide or with a nucleic acid fragment). A sharp peak of radioactivity found in the position of bromophenol blue, mentioned in an earlier publication (van der Walt et al., 1982), was largely due to residual nonincorporated radioactivity which is difficult to remove completely from the gel by washing (Materials and Methods).

The fact that, in cross-competition experiments with a 10-fold molar excess of unlabeled hormone, T_3 was only about twice as efficient as T_4 as a competitor (Table I) may appear surprising in view of the known ~8-fold higher affinity of the nuclear receptors for T_3 than for T_4 (Koerner et al., 1975). However, the latter affinity ratio holds true only in the presence of thiols, while in their absence the affinity for T_3 is decreased (Torresani & DeGroot, 1975) and that for T_4 is increased (Dozin et al., 1981). As discussed above (Results), omission of thiols is mandatory in photoaffinity labeling experiments in order to ensure efficient covalent labeling. Hence, it is meaningless to compare T_3:T_4 competition ratios derived from photoaffinity labeling experiments with T_3:T_4 affinity ratios derived from Scatchard analysis where thiols are present. This does not invalidate competition data obtained by photoaffinity labeling where covalent incorporation of the labeled hormone is, on account of the irreversibility of covalent attachment, a function not only of affinity but also of binding capacity. Such data allow a distinction between proteins with high-affinity, low-capacity sites and other ligand binding proteins. Since receptors are characterized by high-affinity and low-capacity sites for the specific ligand, it must be concluded that those binding proteins which exhibit the highest degree of inhibition of covalent labeling when a small excess of unlabeled over labeled ligand is used are receptors. Consequently, the 56- and 45-kDa proteins have the characteristics of receptors.

The similar behavior of the 56- and 45-kDa proteins seen in competition experiments (Table I) suggested a possible structural relationship between these two proteins. Amino acid sequence analogies were indeed revealed by the nearly identical radioactivity profiles obtained after partial digestion with *Staphylococcus aureus* V8 protease and subsequent SDS gel electrophoresis (Figure 3). The proteolytic digestion had purposely not been carried out to completion in order to obtain enough intermediary peptides for more precise comparison. After completion of these experiments, Pascual et al. (1982) reported similarities between the 57- and 47-kDa thyroid binding proteins in GH_1 cell nuclei. This observation is in agreement with our data although these authors compared limit digests generated with up to 50-fold higher concentrations of *Staphylococcus aureus* V8 protease than those used in our assay.

The presence in nuclei of two receptors having similar amino acid sequences is compatible with various possible mechanisms: (1) both proteins are independently synthesized in vivo; (2) only the 56-kDa protein is synthesized and then undergoes proteolytic degradation either in vivo or in vitro to form the 45-kDa protein; (3) only the 45-kDa protein is synthesized and then forms the 56-kDa protein by posttranslational modification in vivo. It has been suggested (Pascual et al., 1982) that the 56-kDa protein may have arisen by zero-length protein–protein cross-linking of the 45-kDa protein under the influence of UV light (Kunkel et al., 1981). Such a hypothesis is not valid in view of the fact that the 56-kDa protein was also observed in nuclear extracts in the absence of UV light after affinity labeling with the bromoacetyl derivatives of the thyroid hormones (Nikodem et al., 1980).

In conclusion, using our method of photoaffinity labeling with the underivatized thyroid hormones, we (1) identified two nuclear receptors for T_4 and T_3 in rat liver nuclear extract (the 56- and 45-kDa proteins); (2) detected the presence in the nuclei of two additional thyroid hormone binding proteins (the 96- and 35-kDa proteins), and (3) demonstrated a structural relationship between the 56- and 45-kDa receptor proteins. Since our methodology permits discrimination between receptors and other hormone binding proteins in spite of the irreversibility of photoaffinity labeling, it can be applied to the characterization of thyroid hormone binding sites in different tissues and to the analysis of their distribution among different subcellular fractions (Dozin et al., 1985). Moreover, since many thyroid hormone effects are belived to be initiated at the nuclear receptor level and to involve control of gene transcription, the covalent photoattachment of the hormone to its receptor(s) should allow localization of nucleotide sequences on T_3-regulated genes.

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