Comparative Characterization of Thyroid Hormone Receptors and Binding Proteins in Rat Liver Nucleus, Plasma Membrane, and Cytosol by Photoaffinity Labeling with L-Thyroxine

Beatrice Dozin,‡ Hans J. Cahnmann, and Vera M. Nikodem*

Clinical Endocrinology Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

Received August 13, 1984

ABSTRACT: Photoaffinity labeling with underivatized thyroxine (T_4) was used to identify and compare the T_4 binding proteins in rat liver cytosol, nuclear extract, and purified plasma membrane. When these subcellular fractions were incubated with a tracer concentration of [^{125}I]T_4, irradiated with light above 300 nm, and individually analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the radioactivity profiles revealed the presence of T_4 binding proteins of molecular masses of 70, 52, 43, 37, 30, and 26 kilodaltons (kDa) in cytosol, of 96, 56, 45, and 35 kDa in nuclear extract, and of 70, 44, and 30 kDa in plasma membrane. Competition experiments performed in the presence of a 1000-fold excess of unlabeled T_4 demonstrated that these binding proteins display different hormone binding activities. The similar electrophoretic mobilities of some binding proteins present in the different subcellular fractions, i.e., the 70-, 43–45-, and 30-kDa proteins, suggested that these proteins might be identical. However, double-labeling experiments in which plasma membrane, nuclear extract, and cytosol were photolabeled with either [^{125}I]- or [^{131}I]T_4 and mixed, two at a time, in all possible combinations showed that from one cellular fraction to another, the radioactivity peaks corresponding to the ~70-, 43–45-, and 30-kDa proteins were not superimposed. Their relative positions on the gel differed by one or two slices, which indicated differences in molecular mass of 1.9–3.6 kDa. Moreover, enzymatic digestion with *Staphylococcus aureus* V8 protease of these three proteins, prepared from each subcellular fraction, yielded dissimilar peptide patterns. It is concluded that the T_4 binding proteins present in nuclear extract, plasma membrane, and cytosol differ not only in their respective hormone binding activities but also in their molecular masses.

Thyroid hormones are known to affect many tissues and to stimulate multiple processes of cellular metabolism, which raises the question as to whether all effects result from the induction of a common primary event or correspond to different responses elicited by the hormone at several cellular levels. Although thyroid hormone action is believed to be initiated in the nucleus through the binding of the hormone to chromatin-linked receptors (Oppenheimer, 1975; Baxter et al., 1979; Samuels, 1983), extranuclear binding sites have been described in several subcellular compartments including plasma membrane (Pliam & Goldfine, 1977; Gharbi & Torresani, 1979; Holm & Jacquemin, 1979; Krenning et al., 1979, 1981; Gharbi-Chihi & Torresani, 1981; Horiuchi et al., 1982; Segal & Ingbar, 1982; Botta et al., 1983), mitochondria (Sterling et al., 1978), and cytosol (Davis et al., 1974; Dillmann et al., 1974; Samuels et al., 1974; Sterling et al., 1974; Defer et al., 1975; Yoshizato et al., 1975; Hamada & Fukase, 1976; Visser et al., 1976; Galton, 1977, 1980; Geel, 1977; Yoshida & Davis, 1977; Dozin & De Nayer, 1978; Lennon et al., 1980; Geel et al., 1981). It has been suggested that these additional binding sites may be involved in some extranuclear early responses to thyroid hormone stimulation. However, it remains unknown whether or not they constitute physiologically independent or interacting receptors, and whether or not they are related to the nuclear binding site.

Two orders of saturable binding sites for 3,5,3′-triiodo-L-thyronine (T_3)[1] and/or T_4 were identified in purified plasma membranes from rat liver (Pliam & Goldfine, 1977; Gharbi-Chihi & Torresani, 1981), kidney (Gharbi-Chihi & Torresani, 1981), erythrocytes (Botta et al., 1983), thymocytes (Segal & Ingbar, 1982), and human cell ghosts (Holm & Jacquemin, 1979). Values for the dissociation constant (K_d) ranged from 0.39 to 3.2 nM for the high-affinity, low-capacity system and from 25 to 220 nM for the low-affinity, high-capacity component. The uptake of both T_3 and T_4 by the high-affinity sites was shown to be energy (ATP) dependent (blocked by metabolic inhibitors such as KCN, 2,6-dinitrophenol, and oligomycin) (Krenning et al., 1981) and inhibited by sulfhydryl-blocking agents, proteases, and phospholipase A (Gharbi & Torresani, 1979). Using affinity labeling with the derivative *N*-(bromoacetyl)-T_3, Horiuchi et al. (1982) identified three binding proteins with molecular masses of 55, 47, and 33 kDa, respectively, in the GH_3 cell plasma membrane. Saturable cytosolic binding proteins for T_4 and T_3 were described in many amphibian as well as mammalian tissues (Davis et al., 1974; Dillmann et al., 1974; Samuels et al., 1974; Sterling et al., 1974; Defer et al., 1975; Yoshizato et al., 1975; Hamada & Fukase, 1976; Visser et al., 1976; Galton, 1977, 1980; Geel, 1977; Hamada et al., 1977; Yoshida & Davis, 1977; Dozin & De Nayer, 1978; Lennon et al., 1980; Geel et al., 1981). Various numbers of classes of binding sites ranging from 1 to 5 were reported. The dissociation constant values were on the order of 0.53–13.5 nM for T_4 and 15–55 nM for

* Address correspondence to this author at the Department of Health, Education and Welfare, National Institutes of Health.

‡ Recipient of a Fogarty Postdoctoral Fellowship from the National Institutes of Health.

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

T_3. No molecular sizes were estimated for the different cytosolic binding proteins except for the values of 53 and 70 kDa ascribed to the single peak of binding activity recovered from dog liver cytosol (Davis et al., 1974) and rat cerebral cortex cytosol (Geel et al., 1981), respectively, by Sephadex G-100 chromatography.

It appears from the above-mentioned equilibrium studies that both membrane and cytosolic binding sites differ from the nuclear receptors by their respective affinities for thyroid hormones (Samuels et al., 1974; DeGroot & Torresani, 1975; Baxter et al., 1979), the latter being characterized by a higher affinity constant on the order of 10^{10} M^{-1}. Regarding the number of classes of binding proteins in the different subcellular compartments, no accurate comparison could be made on the basis of the Scatchard plot analysis commonly used in those studies since it does not allow distinction between binding proteins sharing similar affinities and/or binding capacities. Therefore, we used another approach, photoaffinity labeling with the underivatized hormones, for the characterization of the T_3 and T_4 receptors in rat liver nuclei (Dozin et al., 1985). Four nuclear binding proteins with molecular masses of 96, 56, 45, and 35 kDa, respectively, were identified. By competition experiments, the 56- and 45-kDa proteins were recognized as thyroid hormone receptors. Enzymatic digestion of these two proteins with *Staphylococcus aureus* V8 protease indicated that they share homologous amino acid sequences.

In the present study, we probed the distribution, the size, and the binding activity of T_4 binding proteins in rat liver plasma membrane and cytosol using the technique of covalent photoattachment followed by SDS–polyacrylamide gel electrophoresis. We then compared them with the nuclear binding proteins.

Materials and Methods

Reagents. Carrier-free [3′,5′-^{125}I]T_4 (5500 μCi/μg) and [3′,5′-^{131}I]T_4 (4540 μCi/μg) were purchased from New England Nuclear. T_4 was obtained from Glaxo Laboratories and then further purified according to Kerwin (1972). *Staphylococcus aureus* V8 protease was from Miles Laboratories.

Nuclear Extract: Preparation and Photoaffinity Labeling. Nuclei were prepared from rat liver according to Blobel & Potter (1966) with the modifications described previously (Dozin et al., 1985). After irradiation, the photolabeled nuclear proteins were analyzed by 10% SDS–polyacrylamide slab gel electrophoresis.

Plasma Membrane: Purification and Photoaffinity Labeling. Purification of rat liver plasma membrane was performed according to Neville (1968). On the basis of adenylate cyclase activity, determined by the procedure of Salomon (1979), the membrane preparation was enriched 17-fold over the total tissue homogenate. The membranes were washed twice with 20 mM Tris-HCl, pH 7.6, containing 0.2 M sucrose, 10 mM NaCl, 1 mM $MgCl_2$, and 2 mM EDTA. Membrane proteins were solubilized with 1% Triton X-100 for 15 min at 4 °C. The mixture was diluted with the washing buffer to a final concentration of 0.1% Triton. Membrane proteins (150 μg) were then incubated with 1 nM [^{125}I]T_4 in a final volume of 250 μL for 3 h at 4 °C. A 1000-fold excess of unlabeled T_4 was added to a parallel incubation sample. At equilibrium, the samples were irradiated for 50 min in the same manner as the nuclear extract. The photolabeled proteins were then resolved on a 10% SDS–polyacrylamide slab gel.

Cytosol: Preparation and Photoaffinity Labeling. The entire procedure was performed at 0–4 °C. Rat liver was perfused in situ through the portal vein with buffered phosphate saline, pH 7.3 (Dulbecco), to prevent contamination of the cytosolic fraction with serum binding proteins for thyroid hormones. The tissue was homogenized in 5 volumes of 20 mM Tris-HCl, pH 7.6, containing 0.32 M sucrose and 1 mM $MgCl_2$. The homogenate was centrifuged at 8000*g* for 15 min, and the supernatant was clarified by centrifugation at 109000*g* for 60 min. The upper lipid layer was discarded, and the cytosolic supernatant (180 μg of protein in 250 μL final volume) was incubated with [^{125}I]T_4 alone or in the presence of a 1000-fold excess of unlabeled hormone. In view of the known lower affinity of the cytosolic binding sites for the thyroid hormones, as compared with the membrane binding proteins and the nuclear receptors (Dillmann et al., 1974), 5 nM labeled T_4 was used. After a 75-min incubation, the samples were irradiated for 50 min under the same conditions as described above for the membrane and nuclear preparations and then analyzed on a 10% SDS–polyacrylamide slab gel.

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide slab gel electrophoresis was performed according to Laemmli (1970) as described previously (Dozin et al., 1985).

Double Photoaffinity Labeling with [^{125}I]- and [^{131}I]T_4. Cytosolic proteins were incubated with 5 nM [^{131}I]T_4, plasma membrane with 1 nM [^{125}I]T_4, and nuclear extract with either 1 nM [^{125}I]T_4 or 1 nM [^{131}I]T_4. After irradiation, the subcellular fractions were mixed, two at a time, in the following combinations: ^{131}I-photolabeled cytosol was mixed with nuclear or membrane proteins photolabeled with [^{125}I]T_4; ^{131}I-photolabeled nuclear extract was combined with ^{125}I-photolabeled plasma membrane. The samples were then electrophoresed on a SDS–polyacrylamide gel as described above. After the radioactivity in each gel slice was counted, the cpm were corrected for the spillover (15%) of ^{131}I into the ^{125}I channel.

Proteolytic Digestion of Photolabeled Nuclear, Cytosolic, and Membrane Proteins. Enzymatic digestion with *Staphylococcus aureus* V8 protease was performed according to Cleveland et al. (1977). Nuclear, cytosolic, and membrane proteins were photolabeled with [^{125}I]T_4 and resolved on a 10% SDS–polyacrylamide slab gel. Slices containing labeled membrane proteins of 70, 44, and 30 kDa, cytosolic proteins of 70, 43, and 30 kDa, and nuclear proteins of 45 kDa were placed individually in separate slots of a 17% SDS–polyacrylamide gel, overlaid with a solution of 1, 2.5, or 5 μg of *Staphylococcus aureus* V8 protease in 15 μL of sample buffer (Laemmli, 1970), and electrophoresed. When the bromophenol blue had migrated ~2 cm into the stacking gel, the power was turned off temporarily (30 min) to allow proteolysis to proceed. After electrophoresis, the gels were stained, destained, dried, and exposed for 2 days to a Kodak XAR2 film using an intensifying screen.

Results

Identification of T_4 Binding Proteins in Rat Liver Plasma Membrane, Cytosol, and Nuclear Extract. T_4 binding proteins were identified in plasma membrane, cytosol, and nuclear extract after photolabeling with [^{125}I]T_4 by SDS–polyacrylamide gel electrophoresis. Figure 1A depicts the radioactivity profile obtained with purified plasma membrane, showing three binding proteins of molecular masses of 70, 44, and 30 kDa. The distribution of the hormone among these proteins, as determined by the total radioactivity under each peak, was 18%, 43%, and 39%, respectively. The degree of specificity of the binding of the hormone to each protein was assessed by competition experiments. The membrane preparation was incubated with 1 nM [^{125}I]T_4 alone or in the presence of a 1000-fold excess of unlabeled T_4. This excess of competitor inhibited the covalent incorporation of radioactivity in the

Table I: Competition of [^{125}I]T_4 with a 1000-fold Excess of Unlabeled T_4 for Binding to Proteins in Different Subcellular Fractions[a]

subcellular fraction	hormone binding protein (kDa)	inhibition[b] (%)	n[d]
plasma membrane	70	46 ± 2.6[c]	3
	44	72 ± 3.2	
	30	57 ± 4.2	
cytosol	70	55 ± 2.6	4
	52	55 ± 6.4	
	43	59 ± 6.2	
	37	42 ± 5.2	
	30	72 ± 4.3	
	26	10 ± 2.8	
nuclear extract	96	40 ± 9.1	3
	56	75 ± 4.6	
	45	78 ± 2.1	
	35	30 ± 1.0	

[a] Plasma membrane and nuclear extract were incubated with 1 nM [^{125}I]T_4 alone or in the presence of 1 μM unlabeled T_4. Cytosol was incubated with 5 nM [^{125}I]T_4 alone or in the presence of 5 μM unlabeled T_4. Incubation was followed by irradiation and SDS–polyacrylamide gel electrophoresis as described in the legend to Figure 1. [b] Inhibition of photoattachment of labeled T_4 expressed as a percentage of the radioactivity measured in the absence of unlabeled competitor. [c] Mean value ± SD. [d] Number of experiments per subcellular fraction.

44-kDa protein to a greater extent than in the 70- and 30-kDa proteins (Figure 1A and Table I). The inhibition was 72% for the former and 46% and 57%, respectively, for the latter two. It should be kept in mind that the degrees of inhibition of the covalent incorporation of [^{125}I]T_4 in the binding proteins depend on both their affinity and their binding capacity for the hormone and therefore reflect their respective binding activities. Our data indicate that the 70- and 30-kDa proteins, although being T_4 binding proteins, display lower binding activities for the hormone than the 44-kDa protein.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of covalently labeled cytosol revealed six distinct T_4 binding proteins among which the hormone was nearly equally distributed (Figure 1B). The respective molecular masses of these proteins were 70, 52, 43, 37, 30, and 26 kDa. Virtually identical radioactivity profiles were obtained when 1 or 5 nM [^{125}I]T_4 was used. We routinely used 5 nM labeled hormone to incorporate enough radioactivity for accurate evaluation in the competition experiments. By these competition assays, the 30-kDa protein was shown to have the highest specificity for T_4 (Table I). The photoattachment of the tracer to this protein was inhibited by 72% while for most of the other proteins the inhibition varied from 42% to 59%. The 26-kDa protein appeared either to be nonspecific or to have low-affinity, high-capacity site(s) for the hormone since only a minimal inhibition (~10%) was observed. A comparison of the binding proteins in cytosol and plasma membrane shows that, in both compartments, the ~70-kDa proteins have similar binding activities for T_4 while the cytosolic 30-kDa protein appears much more specific for the hormone than the corresponding membrane protein (Table I).

Figure 1C illustrates the typical radioactivity profile obtained after photoaffinity labeling of a nuclear extract with [^{125}I]T_4. As reported previously (Dozin et al., 1985), T_4 was mainly incorporated in the 56- and 45-kDa proteins and to a lesser extent in the 96- and 35-kDa proteins. In the presence of a 1000-fold excess of unlabeled T_4, the covalent incorporation of radioactivity in the 56- and 45-kDa proteins was strongly inhibited (75% and 78%, respectively). The degrees of inhibition observed with the two other proteins were much less: 40% for the 96-kDa protein and 30% in the case of the 35-kDa protein (Table I). This indicated that the 56- and

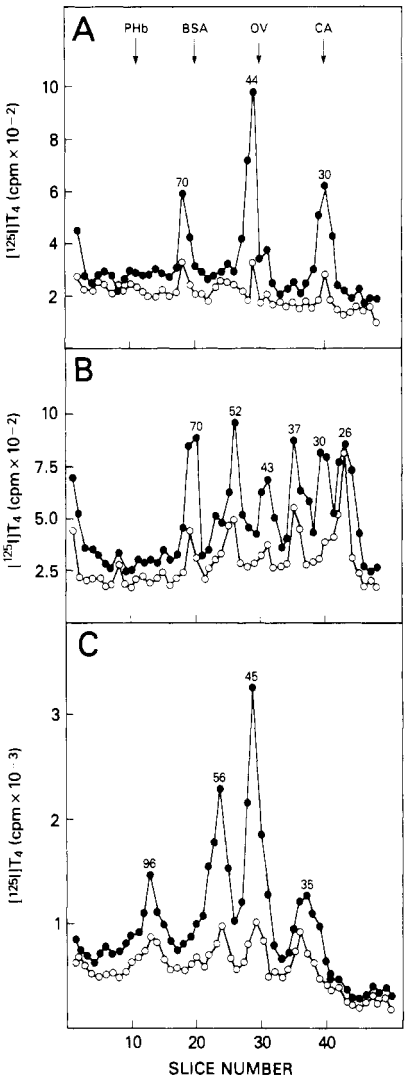

FIGURE 1: SDS–polyacrylamide gel electrophoresis of rat liver proteins photolabeled with [^{125}I]T_4. Purified plasma membrane (150 μg of protein) (A), cytosol (180 μg of protein) (B), and nuclear extract (120 μg of protein) (C) were incubated with 1, 5, and 1 nM [^{125}I]T_4, respectively, alone (●) as well as in the presence of a 1000-fold excess of unlabeled T_4 (○). At equilibrium, the samples were irradiated with light above 300 nm and electrophoresed on a 10% SDS–polyacrylamide gel. After the gel was stained and destained, it was cut in 2-mm slices, and the radioactivity of each slice was counted. Numbers above peaks indicate the molecular masses (in kilodaltons) of the labeled proteins. Marker proteins are phosphorylase *b* (94 kD), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). Their positions are indicated by arrows in panel A only. They deviate slightly in panels B and C from those indicated in panel A.

45-kDa proteins contain high-affinity, low-capacity sites for T_4. Table I also shows that the 45-kDa nuclear protein has approximately the same T_4 binding activity as the plasma membrane protein of similar molecular mass. In contrast, the 43-kDa cytosolic protein shows a lower binding activity for the hormone.

From this set of experiments, we conclude that (1) each of the three subcellular fractions studied, plasma membrane, cytosol, and nuclear extract, contains several T_4 binding proteins and (2) binding proteins of similar molecular masses are present in two or three of the subcellular compartments. This is the case for the ~70- and 30-kDa proteins identified in plasma membrane and cytosol and for the protein in the range of 43–45 kDa detected in all fractions. Although from one subcellular fraction to another these proteins did not always display similar binding activities for T_4 (competition

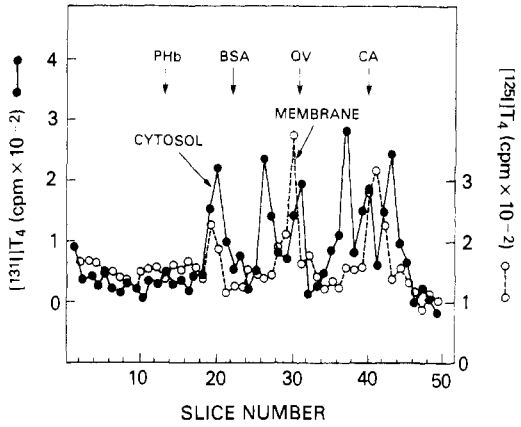

FIGURE 2: SDS–polyacrylamide gel electrophoresis of ^{131}I-labeled rat liver cytosol and ^{125}I-labeled rat liver plasma membrane. Cytosol was incubated with 5 nM [^{131}I]T_4 and plasma membrane with 1 nM [^{125}I]T_4. After irradiation, the two samples were combined and electrophoresed on a 10% SDS–polyacrylamide gel. The gel was then worked up as described in the legend to Figure 1. The cpm were corrected for the spillover of ^{131}I into the ^{125}I channel.

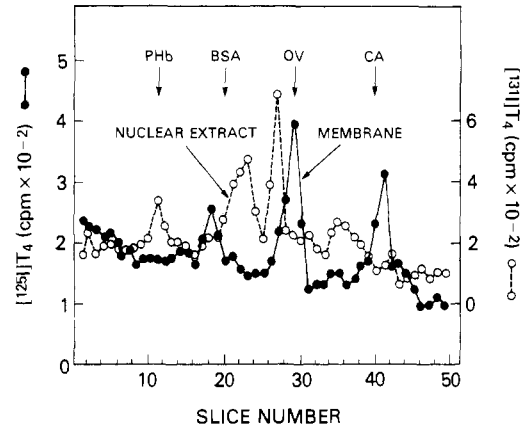

FIGURE 3: SDS–polyacrylamide gel electrophoresis of ^{125}I-labeled rat liver plasma membrane and ^{131}I-labeled rat liver nuclear extracts. Plasma membrane was incubated with 1 nM [^{125}I]T_4 and nuclear extract with 1 nM [^{131}I]T_4. After irradiation, the samples were worked up as described for Figure 2.

studies), we addressed the question as to whether or not they are identical in molecular size and structurally related. Their relative electrophoretic mobilities were precisely determined by double photoaffinity labeling with [^{125}I]- and [^{131}I]T_4. Primary structure analogies were investigated by enzymatic digestion with *Staphylococcus aureus* V8 protease.

Double Photoaffinity Labeling of Plasma Membrane, Cytosol, and Nuclear T_4 Binding Proteins with [^{125}I]- and [^{131}I]T_4. Double-labeling experiments were carried out to determine whether or not the 70-, 43–45-, and 30-kDa proteins present in the different subcellular fractions comigrate when electrophoresed together. For this purpose, cytosol was photolabeled with [^{131}I]T_4, plasma membrane with [^{125}I]T_4, and nuclear extract with either [^{125}I]T_4 or [^{131}I]T_4. The samples were combined, two at a time, in all possible combinations and subjected to SDS–polyacrylamide gel electrophoresis. When [^{131}I]T_4-photolabeled cytosol was electrophoresed together with [^{125}I]T_4-photolabeled plasma membrane, the peaks of radioactivity corresponding to the ~70-, 43–44-, and 30-kDa proteins common to both fractions differed by one gel slice, which suggested the presence of distinct proteins of different molecular masses (Figure 2). According to the standard calibration curve, one gel slice corresponded to a difference in molecular mass of ~1.9 kDa. Figure 3 shows the radioactivity profile obtained after SDS–polyacrylamide gel electrophoresis of [^{125}I]T_4-photolabeled plasma membrane mixed with [^{131}I]T_4-photolabeled nuclear extract. The pattern clearly indicates that the binding proteins of ~44–45 kDa initially detected in both fractions (Figure 1A,1C) are not identical since their respective levels of migration differed by two gel slices. The double-labeling experiment with [^{131}I]T_4-photolabeled cytosol and [^{125}I]T_4-photolabeled nuclear extract shown in Figure 4 ruled out the possibility of an identity between the 45-kDa nuclear protein and the 43-kDa cytosolic binding protein. Indeed, the peaks of radioactivity corresponding to these two proteins were separated by two gel slices, which indicated a difference in their molecular masses of about 3.6 kDa.

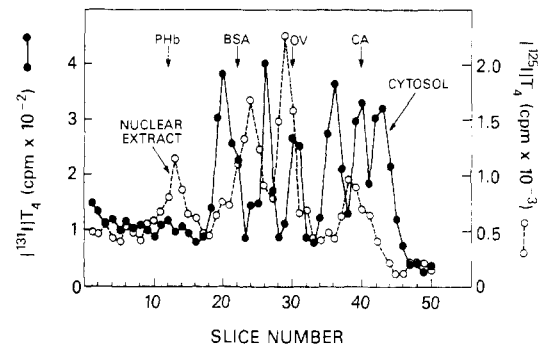

FIGURE 4: SDS–polyacrylamide gel electrophoresis of ^{131}I-labeled rat liver cytosol and ^{125}I-labeled rat liver nuclear extract. Cytosol was incubated with 5 nM [^{131}I]T_4 and nuclear extract with 1 nM [^{125}I]T_4. After irradiation, the samples were worked up as described for Figure 2.

Enzymatic Digestion of [^{125}I]T_4-Photolabeled Plasma Membrane, Nuclear Extract, and Cytosolic Proteins. The double-labeling experiments showed that the T_4 binding proteins of 70, 43–45, and 30 kDa detected in plasma membrane, cytosol, and nuclear extract were not identical in terms of electrophoretic mobility. To further ascertain dissimilarities among these proteins of different subcellular origin, partial proteolytic digestion with *Staphylococcus aureus* V8 protease was performed after photoaffinity labeling of each fraction with [^{125}I]T_4. Figure 5 presents the autoradiograms resulting from SDS–polyacrylamide gel electrophoresis of the digests. Digestion of the 43–45-kDa proteins from nuclear extract, cytosol, and plasma membrane with 1 μg of protease (Figure 5A, lanes 1, 2, and 3, respectively) generated dissimilar peptide patterns. The differences in both the number of intermediary peptides and the relative positions of these peptides on the gel demonstrate the absence of a close structural relationship between these three proteins. This was confirmed by the fact that in the presence of a higher concentration of enzyme (2.5 μg; Figure 5B, lanes 1–3), the smallest fragment derived from the nuclear protein was not generated from either the cytosolic or the membrane protein.

Dissimilar radioactivity patterns were also observed after digestion of the 30-kDa cytosolic and membrane proteins with either 1 or 2.5 μg of *Staphylococcus aureus* V8 protease (Figure 5A,B, lanes 4 and 5). Digestion of the cytosolic protein resulted in five distinct peptides with electrophoretic mobilities different from those of the four fragments generated from the corresponding membrane protein.

When 1 μg of protease was used, the cytosolic 70-kDa protein was fragmented into four to five peptides while the corresponding plasma membrane protein remained intact (Figure 5A, lanes 6 and 7). A strong resistance of the membrane protein to proteolysis observed even in the presence of 5 μg of protease (Figure 5B, lane 8) rendered difficult a definite comparison of possible primary structure analogies

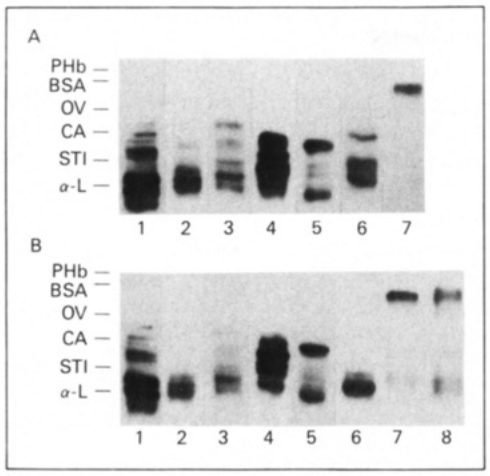

FIGURE 5: Autoradiograms of enzymatic digests of [^{125}I]T_4-photolabeled 70-, 43–45-, and 30-kDa proteins from plasma membrane, cytosol, and nuclear extract. Rat liver plasma membrane and nuclear extract were photolabeled with 1 nM [^{125}I]T_4 and cytosol with 5 nM [^{125}I]T4. The samples were electrophoresed on a 10% SDS–polyacrylamide gel. After the gels were sliced and counted, the slices containing the 70-, 43-, and 30-kDa cystolic proteins, the 70-, 44-, and 30-kDa membrane proteins, and the 45-kDa nuclear protein were placed in different slots of a 17% SDS–polyacrylamide gel and digested with different concentrations of *Staphylococcus aureus* V8 protease as described under Materials and Methods. The gels were stained, destained, dried and exposed for 2 days to Kodak XAR2 film. Marker proteins are phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). (A) Digestion with 1 μg of protease; (B) digestion with 2.5 μg of protease except lane 8 (5 μg); lanes 1, 45-kDa nuclear protein; lanes 2, 43-kDa cytosolic protein; lanes 3, 44-kDa membrane protein; lanes 4, 30-kDa cytosolic protein; lanes 5, 30-kDa membrane protein; lanes 6, 70-kDa cytosolic protein; lanes 7 and 8, 70-kDa membrane protein.

with the cytosolic protein. Nevertheless, this resistance in itself reveals a difference between these two proteins.

Discussion

Using photoaffinity labeling with underivatized T_4, we identified the T_4 binding proteins in rat liver plasma membrane and cytosol and compared them with the four thyroid hormone binding proteins of 96, 56, 45, and 35 kDa which we recently (Dozin et al., 1985) characterized in the nuclear extract. When membrane and cytosolic fractions photolabeled with [^{125}I]T_4 were individually analyzed by SDS–polyacrylamide gel electrophoresis, T_4 binding proteins of molecular masses of 70, 44, and 30 kDa were detected in the former and of 70, 52, 43, 37, 30, and 26 kDa in the latter. Double photoaffinity labeling experiments of nuclear extract, plasma membrane, and cytosol with [^{125}I]- or [^{131}I]T_4 demonstrated that none of the binding proteins with similar sizes found in the different subcellular compartments (i.e., the 70-, 43–45-, and 30-kDa proteins) had identical electrophoretic mobilities. From one subcellular fraction to another, the relative positions of migration of these proteins indicated differences in molecular mass of 1.9–3.6 kDa.

Competition experiments with a 1000-fold excess of unlabeled T_4 were performed to assess the specificity for T_4 of the binding proteins in each subcellular fraction. As pointed out (Reults), the covalent incorporation of [^{125}I]T_4, on account of the irreversibility of photoaffinity labeling, allows an estimation of the overall T_4 binding activity of a given binding protein rather than an accurate quantitation of its affinity and/or binding capacity. Moreover, covalent photoattachment always results in a nonspecific binding slightly higher than that determined under equilibrium conditions with the classical ion-exchange resin method. As discussed previously (Dozin et al., 1985), this may be due to partial conformational alteration of the binding sites during irradiation and/or to adsorption of secondary breakdown products of the tracer to the proteins or the gel itself. Taking these two technical comments into account, we conclude from the competition studies that, from one compartment to another, binding proteins of similar molecular weight display different relative T_4 binding activities.

Both the double-labeling and the competition experiments suggest that the binding proteins of 70, 43–45, and 30 kDa are different in plasma membrane, nuclear extract, and cytosol. Enzymatic digestion of these proteins with *Staphylococcus aureus* V8 protease clearly demonstrated their structural dissimilarity.

The presence of several distinct T_4 binding proteins among different subcellular compartments raises the question of their physiological significance. According to the theory of the initiation of thyroid hormone action at the nuclear level, the two highly specific binding proteins identified in the nuclear extract, i.e., the 56- and 45-kDa proteins, can be classified as thyroid hormone receptors. This terminology is legitimate since the binding of the hormone to high-affinity, low-capacity chromatin-linked binding proteins has been shown to be followed by biological responses. For example, the analysis of the correlation between the nuclear receptor occupancy by T_3 and the stimulation of rat liver malic enzyme demonstrated that a maximal enzyme induction was maintained as long as the nuclear sites were saturated by the hormone (Oppenheimer et al., 1975).

The physiological implications of thyroid hormone binding sites at the plasma membrane level are still unknown. The presence of high-affinity, low-capacity membrane binding sites suggests a carrier-mediated translocation of the hormone into the cell. The recent vizualization of a clustering and subsequent internalization of tetramethylrhodamine-T_3 in cultured fibroblasts strengthens the hypothesis of a receptor-mediated uptake of the hormone (Cheng et al., 1980). In this respect, it is of interest to mention that no high-affinity, low-capacity membrane binding sites for T_4 and T_3 could be detected in tissues known to be unresponsive to thyroid hormones, such as testis and spleen (Gharbi-Chihi & Torresani, 1981). Besides their binding to specific membrane proteins, thyroid hormones have been shown to elicit in vitro some cellular responses at the plasma membrane level. At physiological concentrations, T_3 and T_4 increase the uptake of two unmetabolized amino acids, cycloleucine and α-aminoisobutyric acid, by rat thymocytes (Goldfine et al., 1975a,b; Etzkorn et al., 1979) and of the glucose derivatives 2-deoxy-D-glucose and 3-*O*-methylglucose by chick embryo heart cells and rat thymocytes (Segal & Ingbar, 1979; Dickstein et al., 1983). These effects can be observed within a few minutes of thyroid hormone stimulation and are not inhibited by cycloheximide, puromycin, or actinomycin D (Segal & Ingbar, 1979; Dickstein et al., 1983), which indicates that they are independent of protein and RNA synthesis. It remains to be demonstrated whether or not these extranuclear effects of the thyroid hormones are linked to the specific binding of T_3 or T_4 to the plasma membrane.

No direct effect of either T_3 or T_4 at the cytosol level has been so far shown to result from the interaction of the hormones with the cytosolic binding sites. The possibility of a physiological relationship between the cytosolic binding proteins and either the plasma membrane or the nuclear sites has been investigated. From these studies, it appears that the cellular uptake of T_3 is not mediated by cytoplasmic proteins

(Eckel et al., 1979). It has also been demonstrated that an initial interaction between thyroid hormones and specific cytosolic proteins is not a prerequisite for translocation of the hormones to the nuclear binding sites (Surks et al., 1975; Tata, 1975). The biological function of the cytosolic binding proteins for thyroid hormones thus remains a matter of speculation. These proteins are commonly believed to play a passive role of transport analogous to that of the binding proteins in serum. According to this concept, they would ensure the intracellular storage of thyroid hormones, the maintenance of a constant and readily available pool of free hormone, and the distribution of the hormone among the different subcellular compartments. It has also been suggested that the cytosolic binding sites could be involved in the intracellular deiodination of T_4 and therefore determine the hormonal turnover within the cells (Samuels et al., 1974).

In summary, our study provides direct evidence for the presence, in different subcellular compartments, of multiple T_4 binding proteins distinguishable by their respective molecular sizes and hormone binding activities. In view of the covalent attachment of the hormone, the method of photoaffinity labeling should be a useful tool in the purification of these binding proteins and the characterization of their binding site(s), as well as in studies of the kinetics of thyroid hormone transport within the cell.

ACKNOWLEDGMENTS

We are grateful to Dr. J. E. Rall for valuable discussion throughout this work and to J. Todd and D. Neely for preparing the manuscript.

Registry No. L-T_4, 51-48-9.

REFERENCES

Baxter, J. D., Eberhardt, N. L., Apriletti, J. W., Johnson, L. K., Ivarie, R. D., Schachter, B. S., Morris, J. A., Seeburg, P. H., Goodman, H. M., Lathman, K. R., Polansky, J. R., & Martial, J. A. (1979) *Recent Prog. Horm. Res. 35*, 97–153.

Blobel, G., & Potter, V. R. (1966) *Science (Washington, D.C.) 154*, 1662–1665.

Botta, J. A., de Mendozas, D., Morero, R. D., & Farias, R. N. (1983) *J. Biol. Chem. 258*, 6690–6692.

Cheng, S. Y., Maxfield, S. R., Robbins, J., Willingham, M. C., & Pastan, I. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3425–3429.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Davis, P. J., Handwerger, B. S., & Glaser, F. (1974) *J. Biol. Chem. 249*, 6208–6217.

Defer, N., Dastugue, B., Sabatier, M. M., Thomopoulos, P., & Kruh, J. (1975) *Biochem. Biophys. Res. Commun. 67*, 995–1004.

DeGroot, L. J., & Torresani, J. (1975) *Endocrinology (Philadelphia) 96*, 357–369.

Dickstein, H., Schwartz, H., Gross, J., & Gordon, A. (1983) *Endocrinology (Baltimore) 113*, 391–398.

Dillmann, W. H., Surks, M. I., & Oppenheimer, J. H. (1974) *Endocrinology (Philadelphia) 95*, 492–498.

Dozin, B., & De Nayer, Ph. (1978) *FEBS Lett. 96*, 152–154.

Dozin, B., Cahnmann, H. J., & Nikodem, V. M. (1985) *Biochemistry* (preceding paper in this issue).

Eckel, J., Rao, G. S., Rao, M. L., & Breuer, H. (1979) *Biochem. J. 182*, 473–491.

Etzkorn, J., Hopkins, P., Gray, J., Segal, J., & Ingbar, S. H. (1979) *J. Clin. Invest. 63*, 1172–1180.

Galton, V. A. (1977) *Acta Endocrinol. (Copenhagen) 85*, 256–266.

Galton, V. A. (1980) *Endocrinology (Baltimore) 107*, 61–69.

Geel, S. E. (1977) *Nature (London) 269*, 428–430.

Geel, S. E., Gonzales, L., & Timiras, P. S. (1981) *Endocr. Res. Commun. 8*, 1–18.

Gharbi, J., & Torresani, J. (1979) *Biochem. Biophys. Res. Commun. 88*, 170–177.

Gharbi-Chihi, J., & Torresani, J. (1981) *J. Endocrinol. Invest. 4*, 177–183.

Goldfine, I. D., Simons, C. G., & Ingbar, S. H. (1975a) *Endocrinology (Philadelphia) 96*, 802–805.

Goldfine, I. D., Simons, C. G., Smith, G. T., & Ingbar, S. H. (1975b) *Endocrinology (Phildelphia) 96*, 1030–1037.

Hamada, S., & Fukase, M. (1976) *J. Clin. Endocrinol. Metab. 42*, 302–308.

Hamada, S., Nanno, M., & Nakamura, H. (1977) *J. Clin. Endocrinol. Metab. 45*, 833–836.

Holm, A. C., & Jacquemin, C. (1979) *Biochem. Biophys. Res. Commun. 89*, 1006–1017.

Horiuchi, R., Johnson, M. L., Willingham, M. C., Pastan, I. H., & Cheng, S. Y. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5527–5531.

Kerwin, J. F., quoted by Cahnmann, H. J. (1972) in *Methods in Investigative and Diagnostic Endocrinology* (Berson, S. A., Ed.) Vol. 1, Part 1, pp 27–51, North-Holland Publishing Co., Amsterdam.

Krenning, E., Docter, R., Bernard, H. F., Visser, T. J., & Hennemann, G. (1979) *FEBS Lett. 107*, 227–230.

Krenning, E., Docter, R., Bernard, B., Visser, T. J., & Hennemann, G. (1981) *Biochim. Biophys. Acta 676*, 314–320.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lennon, A. M., Osty, J., & Nunez, J. (1980) *Mol. Cell. Endocrinol. 18*, 201–214.

Neville, D. M., Jr. (1968) *Biochim. Biophys. Acta 154*, 540–552.

Oppenheimer, J. H. (1975) *N. Engl. J. Med. 292*, 1063–1068.

Oppenheimer, J. H., Schwartz, H. L., & Surks, M. I (1975) *Endocr. Res. Commun. 2*, 309–325.

Pliam, N. B., & Goldfine, I. D. (1977) *Biochem. Biophys. Res. Commun. 79*, 166–172.

Salomon, Y. (1979) *Adv. Cyclic Nucleotide Res. 10*, 35–55.

Samuels, H. H. (1983) in *Molecular Basis of Thyroid Hormone Action* (Oppenheimer, J. H., & Samuels, H. H., Eds.) pp 35–65, Academic Press, New York.

Samuels, H. H., Tsai, J. S., Casanova, J., & Stanley, F. (1974) *J. Clin. Invest. 54*, 853–865.

Segal, J., & Ingbar, S. H. (1979) *J. Clin. Invest. 63*, 507–515.

Segal, J., & Ingbar, S. H. (1982) *J. Clin. Invest. 70*, 919–926.

Segal, J., Schwartz, H., & Gordon, A. (1977) *Endocrinology (Philadelphia) 101*, 143–149.

Sterling, K., Saldanha, V. F., Brenner, M. A., & Milch, P. O. (1974) *Nature (London) 250*, 661–663.

Sterling, K., Lazarus, J. H., Milch, P. O., Sakurada, T. & Brenner, M. A. (1978) *Science (Washington, D.C.) 201*, 1126–1129.

Surks, M. I., Koerner, D. H., & Oppenheimer, J. H. (1975) *J. Clin. Invest. 55*, 50–60.

Tata, J. R. (1975) *Nature (London) 257*, 18–22.

Visser, T. J., Bernard, H. F., Docter, R., & Hennemann, G. (1976) *Acta Endocrinol. (Copenhagen) 82*, 98–104.

Yoshida, K., & Davis, P. J. (1977) *Biochem. Biophys. Res. Commun. 78*, 697–705.

Yoshizato, K., Kistler, A., & Frieden, E. (1975) *J. Biol. Chem. 250*, 8337–8343.