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ARTICLE *in* EUROPEAN JOURNAL OF IMMUNOLOGY · JANUARY 1983

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Jannie Borst, Mary Ann Prendiville and Cox Terhorst[○]

Laboratory of Molecular Immunology, Dana-Farber Cancer Institute, Harvard Medical School, Boston

The T3 complex on human thymus-derived lymphocytes contains two different subunits of 20 kDa*

The human cell surface antigen T3 is involved in several T lymphocyte specific functions, as determined by the effect of monoclonal antibodies (OKT3, anti-Leu-4, UCHT1) directed at this molecular structure. The main target antigen of these monoclonal antibodies is a glycoprotein of 20 kDa. It is associated with four, less predominant, structurally distinct glycoproteins of 25–28 kDa, 37 kDa and 44 kDa. Of these molecules only the 20-kDa T3 antigen could be labeled with the hydrophobic reagent 5-iodonaphthyl-1-azide (INA). Here we present evidence that the main 20-kDa T3 antigen is comprised of, in fact, two structurally different molecules. One of these is a glycoprotein with a protein backbone of 14 kDa, the other is an unglycosylated protein of 20 kDa. This unglycosylated protein is labeled specifically with INA. Additional evidence for the existence of two different 20-kDa T3 antigens is provided by studies using the enzymes endo-β-N-acetylglucosaminidase H and endo-β-N-acetylglucosaminidase F and the drug tunicamycin. We hypothesize that the specific susceptibility to labeling with INA of the unglycosylated 20-kDa T3 form reflects a positioning in the lipid bilayer different from that of the glycosylated 20-kDa T3 form.

1 Introduction

The cell surface antigen T3, recognized by the monoclonal antibody (mAb) reagents OKT3 [1], anti-Leu-4 [2], and UCHT1 [3], is present on all human peripheral blood T lymphocytes, and on mature thymocytes. Anti-Leu-4 and UCHT1 have also been found to react with Purkinje cells in the cerebellum of several species [4], but the target antigen in that tissue has not yet been identified. On thymus-derived lymphocytes, T3 appears to be involved in proliferative functions. Namely, anti-T3 mAb, in picomolar amounts, induce DNA synthesis in T lymphocytes [5]. At higher concentrations, the mAb block T cell proliferative responses to soluble and cell surface antigens [5, 6]. In addition to an involvement in T cell proliferation, the T3 antigen plays a role in target cell lysis by allogeneic cytotoxic T cells [7]. Anti-T3 mAb reagents inhibit the effector function of cytotoxic T lymphocyte clones directed at class I, as well as class II major histocompatibility antigens [8].

The most plausible explanation for the pleiotropic effect of the anti-T3 mAb is that T3 interacts with several functional components in the plasma membrane. Our previous studies support this idea by showing that on T cells, anti-T3 mAb binds to a 20-kDa glycoprotein, which is associated with three other glycoproteins (25–28 kDa, 37 kDa and 44 kDa) [9, 10].

Here, we demonstrate that two 20-kDa T3 species exist. One 20-kDa T3 species, which can be labeled strongly by cell surface radioiodination [11], consists of a 14-kDa polypeptide backbone which carries N-linked oligosaccharides. The other 20-kDa T3 species can be labeled strongly with the hydrophobic reagent 5-iodonaphthyl-1-azide (INA) [12], but only weakly by cell surface radioiodination. Biosynthetic labeling experiments, combined with the use of an inhibitor of glycosylation and the enzymes endo-β-N-acetylglucosaminidase H (Endo-H) [13] and endo-β-N-acetylglucosaminidase F (Endo-F) [14], demonstrated that the INA 20-kDa T3 does not carry asparagine-linked oligosaccharides. We postulate that this hydrophobic 20-kDa T3 species may serve as a nucleus for selective protein-protein interactions on the T cell membrane.

[I 4038]

* This work was supported by grants from the NIH (AI-15066 and AI-17561).

○ Scholar of the Leukemia Society of America.

Correspondence: Jannie Borst, Laboratory of Molecular Immunology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

Abbreviations: **Endo-H, -F:** Endo-β-N-acetylglucosaminidase H, F **IEF:** Isoelectric focusing **INA:** 5-Iodonaphthyl-1-azide **NP40:** Nonidet P-40 **PAGE:** Polyacrylamide gel electrophoresis **PMSF:** Phenyl methyl sulfonyl fluoride **SDS:** Sodium dodecyl sulfate **TEA:** Triethanolamine **TI:** Ovomucoid trypsin inhibitor **2D-GE:** Two-dimensional gel electrophoresis **mAb:** Monoclonal antibody(ies)

2 Materials and methods

2.1 Cells

For all studies described in this report, the T leukemic cell line HPB-ALL [15] was used, which was cultured in RPMI 1640 medium, supplemented with 5% fetal calf serum, at 37 °C, in a 5% CO_2 atmosphere.

2.2 Sera, chemicals

For the isolation of the T3 antigen the anti-Leu-4 mAb was used (a generous gift from Dr. Robert Evans, The Memorial Sloan-Kettering Cancer Center, New York, NY). The precursor for INA, 5-amino-1-azidonaphthalene, was kindly supplied by Dr. Carlos Gitler, The Weizman Institute, Rehovot, Israel. The catalyst for radioiodination, 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (Iodogen) was obtained from Pierce Chemical Co., Rockford, IL. Tunicamycin was purchased from Calbiochem-Behring Corp., La Jolla, CA; Endo-H from Health Research Inc., Albany, NY. Endo-F was generously supplied by Drs. John Elder and Stephen Alexander, Scripps Clinic, La Jolla, CA.

2.3 Labeling procedures

For cell surface radioiodination, 50×10^6 cells from the T leukemic cell line HPB-ALL were labeled with Na[^{125}I] (ICN

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Radiochemicals, Irvine, CA), using 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril as a catalyst [11]. Radioactively labeled INA was prepared on the day of the experiment from 5-amino-1-azidonaphthalene [12]. In a typical experiment, HPB-ALL cells at 2×10^6/ml in phosphate-buffered saline were mixed with 10^9 cpm of [^{125}I]-INA at 4 °C. After 5 min, reduced glutathione was added to a final concentration of 20 mM and cells were irradiated for 3 min with ultraviolet light with wavelengths of approximately 300–400 nm.

Metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine (Amersham Corp., Arlington Heights, IL) was done in RPMI 1640 medium, without methionine and cysteine (Gibco, Grand Island, NY), supplemented with 10% dialyzed fetal calf serum. Cells were preincubated in this medium for 2 h and labeled for 12 h at a concentration of 5×10^6 cells/ml. When tunicamycin was used, it was present at a concentration of 5 μg/ml during both preincubation and labeling. For pulse-chase labeling, cells, resuspended at 50×10^6/ml, were incubated with [^{35}S]methionine and [^{35}S]cysteine for 5 min. Then the culture was diluted fivefold with RPMI 1640 medium containing 0.5 mM cold methionine and cysteine. After 15 min cells were diluted tenfold in icecold phosphate-buffered saline, harvested, and lysed in immunoprecipitation buffer.

2.4 Immunoprecipitation

Radiolabeled HPB-ALL cells were lysed with 1% Nonidet P-40 (NP40) in 0.01 M triethanolamine-HCl (TEA), pH 7.8, 0.15 M NaCl, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 0.02 mg/ml ovomucoid trypsin inhibitor (TI) (TEA/NaCl buffer). Nuclear debris was removed from the lysates by centrifugation at 13 000 × g for 15 min at 4 °C. Further pretreatment of the lysates was done by centrifugation at 100 000 × g for 30 min, and by preclearing with formalin-fixed *Staphylococcus aureus* bacteria (strain Cowan I) and with preformed complexes of mouse immunoglobulin (Ig) and rabbit anti-mouse Ig as described previously [9]. Precleared lysates were incubated for 3–4 h with a preformed complex made with the anti-Leu-4 mAb and rabbit anti-mouse Ig at 4 °C and immunoprecipitates were removed from the lysate by centrifugation at 13 000 × g. Precipitates were resuspended in TEA/NaCl buffer with 0.5% sodium deoxycholate and spun through a discontinuous gradient consisting of one layer of 10% sucrose, 0.5% NP40 in TEA/NaCl buffer, and one layer of 20% sucrose in the same buffer without detergents, in an Eppendorf (Hamburg, FRG) centrifuge at 13 000 × g. Subsequently, the anti-Leu-4 precipitates were washed once in 0.01 M TEA, pH 7.8, containing 1 $MgCl_2$ and 0.5% NP40, followed by a wash in 0.01 M TEA, pH 7.8, 0.2% NP40.

2.5 Enzyme treatments

For neuraminidase treatment, the immune complex was resuspended in 0.05 M sodium acetate, pH 5.5, 0.9% NaCl, 0.1% $CaCl_2$, 1 mM PMSF, 0.02 mg/ml TI and incubated for 4 h at 37 °C. Neuraminidase was added after 0, 30, 60 and 120 min, 2.5 units each time. In control experiments, samples were incubated in the same buffer without the enzyme. For Endo-H treatment, immune complexes were resuspended in 0.15 M sodium citrate, pH 5.5, 1 mM PMSF, 0.02 mg/ml TI, 0.1% sodium dodecyl sulfate (SDS). Endo-H was added at 3 μg/ml and samples were incubated for 3 h at 37 °C [13]. Endo-F treatment was done as described by Elder and Alexander [14]. The immunoprecipitates were resuspended in 0.1 M sodium phosphate buffer, pH 6.1, 50 mM EDTA (disodium salt), 1% 2-mercaptoethanol, 0.1% SDS and boiled for 2 min. NP40 was added to 1% and samples were incubated with Endo-F in the presence of 1 mM PMSF and 0.02 mg/ml TI for 2 h at 37 °C. Samples intended for isoelectric focusing (IEF) were not boiled in the presence of SDS.

2.6 Electrophoresis and autoradiography

SDS polyacrylamide electrophoresis (SDS-PAGE) was carried out on discontinuous vertical slab gels according to a modification of the Laemmli procedure [16]. Gradient gels were regularly made 10–15% in acrylamide. For the second dimension, in two dimensional gel electrophoresis (2D-GE) according to O'Farrell [17], 14–20% gradient gels were used.

For the first dimension in 2D-GE ampholytes (LKB, Bromma, Sweden) of pI 3.5–10, 4–6 and 5–8 were used as 10 : 1 : 1. Samples were taken up in 8.8 M urea, 0.4% SDS, 5% 2-mercaptoethanol, 0.2% ampholytes. After 45 min, an equal volume of 8.8 M urea, 2% NP40, 5% 2-mercaptoethanol, 2% ampholytes was added and electrophoresis was performed. Samples for one dimensional IEF were prepared in an identical fashion and run from cathode to anode on vertical slab gels, containing 8 M urea, 0.5% NP40 and LKB ampholytes of pI 3.5–10, 4–6 and 5–8 as 10 : 1 : 1 [18]. Kodak XAR-5 film was used for autoradiography of ^{125}I-labeled materials in combination with intensifier screens (Cronex Lightning-Plus, Dupont Chemical Co., Newtown, CT). Gels containing proteins labeled with ^{35}S were treated with EN^{3}HANCE (New England Nuclear, Boston, MA) before autoradiography.

3 Results

3.1 [^{125}I]INA reacts specifically with one T3 form

The 20-kDa T3 antigen was isolated from the T leukemic cell line HPB-ALL by immunoprecipitation [9] with the anti-Leu-4 mAb [2]. To obtain the 20-kDa T3 free of the 25–28 kDa, 37 kDa and 44 kDa glycoproteins which are also part of the T3 complex [9, 10], the immune complex was washed with a buffer containing 1 M $MgCl_2$. Since it was previously established that the 20-kDa T3 antigen which is present on the surface of HPB-ALL cells is identical to the one on peripheral blood T lymphocytes [9] and T cell clones [8], only HPB-ALL cells were used in these studies.

The 20-kDa T3 antigens, labeled by three different techniques, were compared by SDS-PAGE (Fig. 1) and IEF (Fig. 2). The techniques used were: labeling with Na^{125}I (cell surface radioiodination) [11], labeling with the hydrophobic reagent [^{125}I]INA [12] and biosynthetic labeling with a mixture of [^{35}S]methionine and [^{35}S]cysteine. INA has a high partition coefficient into membrane lipids. The azide, when activated by ultraviolet light with wavelengths in the range of 300–400 nm, is converted into the reactive nitrene. The nitrene inserts covalently into neighboring membrane components. It appears to insert mainly into intrinsic membrane proteins; only little insertion, if any, takes place into extrinsic proteins [12, 19]. Whereas cell surface radioiodinated and biosynthetically labeled T3 resolved in fuzzy bands upon SDS-PAGE, the

[^{125}I]INA-labeled T3 resolved as a single sharp band (Fig. 1). This indicated that the [^{125}I]INA-labeled T3 might be less heterogeneous than the cell surface radioiodinated or metabolically labeled T3. This was confirmed by IEF (Fig. 2). Of the many differently charged T3 forms that were susceptible to cell surface radioiodination (Fig. 2, lane B), only one form labeled significantly with the [^{125}I]INA reagent (Fig. 2, lane C). The three more basic T3 forms are indicated in Fig. 2 with the letters a, b and c. Only the T3 form at position b is strongly labeled with [^{125}I]INA. The two other forms were also susceptible to labeling with [^{125}I]INA, but to a lesser degree (positions a, c). The labeling intensities of these forms appeared to be variable. The band at pI 5 in Fig. 2, lanes B and C, represents a contaminant, since this band is not always present in anti-Leu-4 immunoprecipitates (see Fig. 2, lanes D–G) and represents a molecule with a molecular mass of about 30 kDa. The three basic T3 forms labeled strongly with ^{35}S-labeled amino acids (Fig. 2, lane A). In contrast, the more acidic T3 molecules were preferentially susceptible to labeling by cell surface radioiodination. These more acidic molecules carry sialic acids and can be converted into forms a, b and c by neuraminidase treatment (Fig. 2, lanes E and G). The IEF pattern of the [^{125}I]INA-labeled T3 was not affected by neuraminidase (Fig. 2, lanes D and F). These labeling experiments indicated that the hydrophobic reagent INA reacts mainly with only one of the 20-kDa T3 forms (position b).

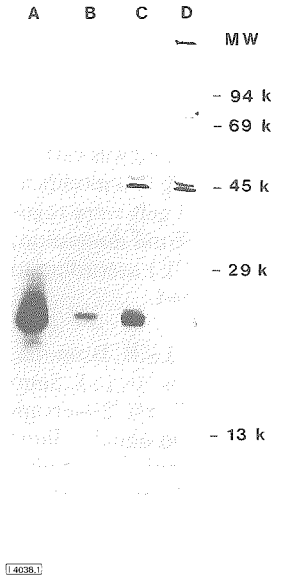

Figure 1. Comparison by SDS-PAGE of the 20-kDa T3 antigen labeled by cell surface radioiodination, with [^{125}I]INA, and with [^{35}S]methionine and [^{35}S]cysteine. HPB-ALL cells were labeled by the various techniques as described in Sect. 2.3, immunoprecipitations were carried out using anti-Leu-4, and immunoprecipitates were analyzed on a 10–15% SDS polyacrylamide gradient gel. (A) T3 labeled by cell surface radioiodination; (B) T3 labeled with [^{125}I]INA; (C) T3 labeled with [^{35}S]methionine and [^{35}S]cysteine; (D) Control precipitate from biosynthetically labeled lysate.

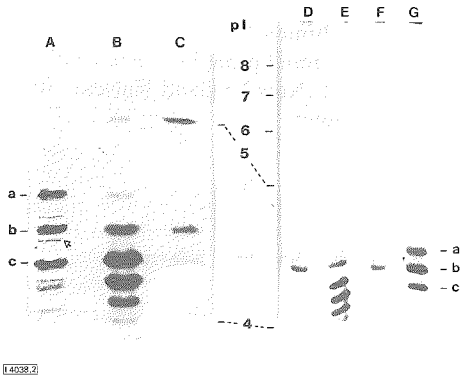

Figure 2. Comparison by IEF of the 20-kDa T3 antigen labeled by various techniques as described in Fig. 1. Anti-Leu-4 immunoprecipitates from HPB-ALL cells, labeled by the three different techniques were analyzed on a IEF slab gel, made as described in Sect. 2.3. In addition, cell surface radioiodinated and [^{125}I]INA-labeled T3 antigens were treated with neuraminidase previous to IEF analysis. (A) T3 labeled with [^{35}S]methionine and [^{35}S]cysteine (the position of actin is indicated by a open arrow); (B) T3 labeled by cell surface radioiodination; (C) T3 labeled with [^{125}I]INA; (D) T3 labeled with [^{125}I]INA, without neuraminidase; (E) T3 labeled by cell surface radioiodination, without neuraminidase; (F) T3 labeled with [^{125}I]INA, with neuraminidase and (G) T3 labeled by cell surface radioiodination, with neuraminidase. The positions of the most basic T3 forms are indicated with the letters a, b, and c.

3.2 The T3 form, labeled by [^{125}I]INA does not carry N-linked carbohydrate side chains

To investigate how the [^{125}I]INA-labeled T3 species was glycosylated, we used a recently described enzyme, Endo-F [14]. Endo-F was found to completely remove both complex and high mannose N-linked oligosaccharides from several well-defined glycoproteins, such as murine leukemia virus glycoprotein gp 70 and HLA-A,-B antigens [14]. As shown in Fig. 3, Endo-F treatment resulted in a reduction in moleculear mass of the cell surface radioiodinated 20 kDa T3 to 14 kDa (Fig. 3, lanes c, d). In contrast, [^{125}I]INA-labeled 20-kDa T3 was not affected at all by the enzyme (Fig. 3, lanes a, b). When the 20-kDa T3 antigen was treated with Endo-F after metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine, only part of the antigen was converted into the 14-kDa product (Fig. 3, lanes e, f). Apparently, the T3 form that is susceptible to labeling with [^{125}I]INA does not carry any asparagine-linked oligosaccharides. The [^{125}I]INA-susceptible form would be represented by the metabolically labeled material that is not sensitive to Endo-F digestion (Fig. 3, lane f).

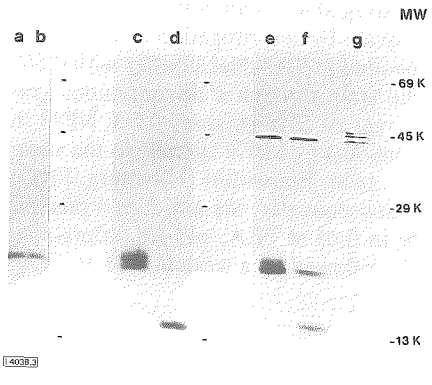

Figure 3. Analysis by SDS-PAGE of 20-kDa T3 antigens treated with Endo-F. HPB-ALL cells were labeled as described in Figs. 1 and 2, and anti-Leu-4 immunoprecipitates were subjected to digestion with Endo-F, or incubated in the absence of enzyme. The protein was recovered by precipitation in 10% trichloroacetic acid and analyzed on 10–15% polyacrylamide gradient gels. (a) T3 labeled with [^{125}I]INA, without Endo-F; (b) T3 labeled with [^{125}I]INA, with Endo-F; (c) T3 labeled by cell surface radioiodination, without Endo-F; (d) T3 labeled by cell surface radioiodination, with Endo-F; (e) T3 labeled with ^{35}S-labeled amino acids, without Endo-F; (f) T3 labeled with ^{35}S-labeled amino acids, with Endo-F and (g) control precipitate from biosynthetically labeled lysate.

Experiments with Endo-H, after pulse-chase labeling, gave comparable results. Transfer of N-linked carbohydrate moieties to the protein backbone is believed to take place cotranslationally [20]. Since conversion of precursor high mannose sugars to mature complex or high mannose forms occurs during the first few hours after translation [20], susceptibility to Endo-H exists during that period. Here, a 5 min pulse with [^{35}S]methionine and [^{35}S]cysteine, followed by a 15 min chase with cold methionine and cysteine, was used. By 2D-GE it was established that there were two T3 forms at position (c). Both were susceptible to Endo-H digestion. In addition, at position (b) there appeared to be two T3 forms of slightly different molecular mass also (Fig. 4A, B). Of these two forms, one was sensitive to digestion with Endo-H, whereas the other was resistant to the enzyme (Fig. 4B, closed arrow). In a control experiment, the precursor of the heavy chain of HLA-A,-B antigens from the same cells was found to be completely susceptible to Endo-H (not shown). This pulse-chase experiment confirms, therefore, the finding that one of the 20-kDa T3 species (at position b) does not contain N-linked oligosaccharides.

Experiments using the drug tunicamycin probided further evidence for the existence of a 20-kDa T3 species which is not glycosylated via asparagine. Tunicamycin inhibits N-linked glycosylation by blocking the coupling of carbohydrate to dolicholphosphate, the carrier molecule that transfers carbohydrate to asparagine in the protein backbone [21]. After biosynthetic labeling in the presence of tunicamycin at 5 μg/ml, a concentration at which glycosylation of HLA-A,-B antigens is completely blocked ([22] and data not shown), a 20-kDa T3 form was still detectable by immunoprecipitation (Fig. 5B, closed arrow). Most of the ^{35}S-labeled amino acids were incorporated in one of the T3 forms found at position b. This form was identified by one-dimensional IEF and by using the coordinates of actin (open arrow) and other background spots. Also in this experiment, the single bands of 20-kDa T3-a and T3-b found in the one-dimensional IEF (Fig. 2), split up into two spots in 2D-GE (Fig. 5A). Therefore, [^{125}I]INA-labeled 20-kDa T3 was analyzed by 2D-GE under identical conditions. As shown in Fig. 5C, the [^{125}I]INA-labeled T3 species appeared at one spot. Mixing experiments with the material from Figs. 5A and B demonstrated that this spot coincides

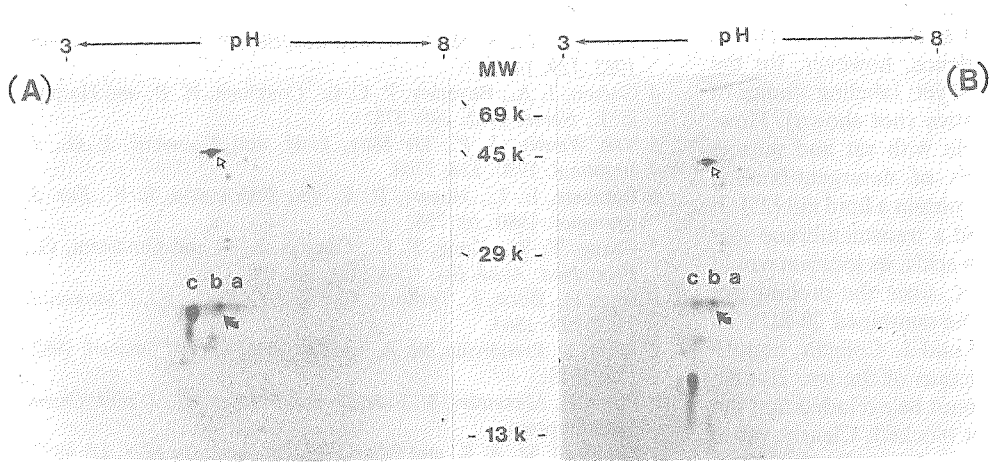

Figure 4. 2D-GE of pulse-labeled T3 antigens after treatment with Endo-H. HPB-ALL cells were pulse labeled for 15 min with a mixture of [^{35}S]methionine and [^{35}S]cysteine as described in Sect. 2.3 and anti-Leu-4 immunoprecipitates were incubated without Endo-H (A), or with Endo-H (B). 2D-GE was carried out according to the method of O'Farrell [17], using a 14–20% SDS polyacrylamide gradient gel in the second dimension. The position of actin is indicated by an open arrow, the position of the T3 form of interest by a closed arrow.

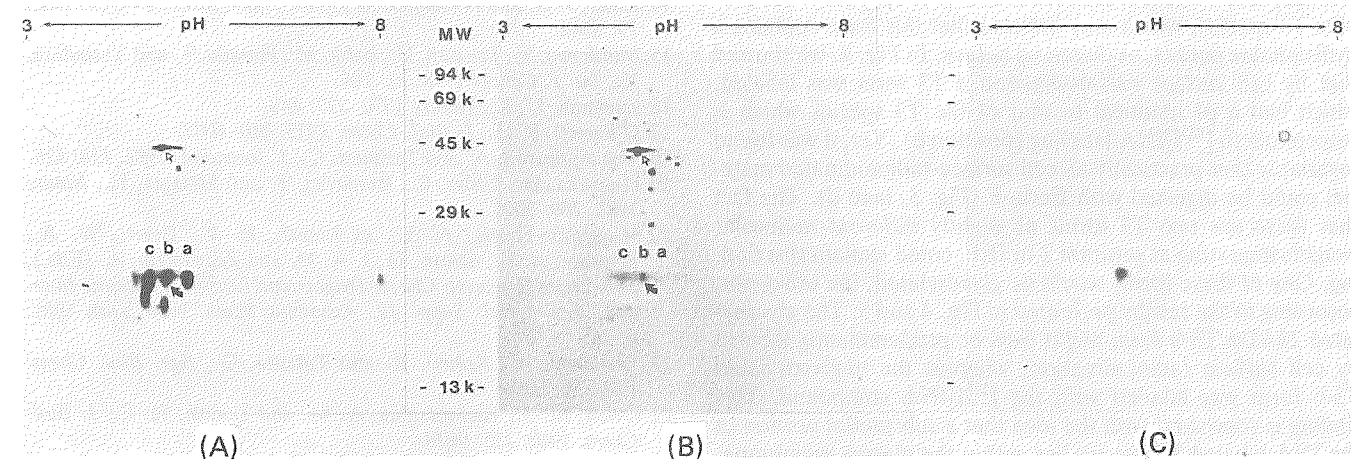

Figure 5. 2D-GE of the 20-kDa T3 antigen, labeled biosynthetically in the presence or absence of tunicamycin, and labeled with [^{125}I]INA. HPB-ALL cells were labeled with ^{35}S-labeled amino acids for 12 h, in the absence of tunicamycin (A), or in the presence of 5 μg/ml tunicamycin (B). (C) shows the T3 antigen, labeled with [^{125}I]INA. 2D-GE was carried out as described in Fig. 4. The position of actin is indicated by an open arrow, the position of the T3 form of interest by a closed arrow.

with the T3 form at position (b) that is indicated by a closed arrow (data not shown). In the presence of tunicamycin apparently only this T3-b form was synthesized.

4 Discussion

All experiments presented here support the idea that two 20-kDa T3 species exist. One species consists of a 14-kDa polypeptide backbone which must carry at least three N-linked oligosaccharides, considering the average size of N-linked sugar moieties [13]. In contrast, the other species does not carry any asparagine-linked carbohydrate. Treatment with trifluoromethane sulfonic acid [23], and alkaline hydrolysis [24] showed that none of the T3 forms contains O-linked oligosaccharides (unpublished results). Therefore, one T3 species has a polypeptide backbone of 14 kDa, the other T3 species, which is specifically labeled with the hydrophobic reagent [^{125}I]INA, has a polypeptide backbone of 20 kDa. Based on the analyses used thus far, we cannot determine whether both forms carry the antigenic determinant recognized by the mAb reagent anti-Leu-4 (OKT3, UCHT1). The most plausible explanation for the existence of these two T3 species is, that the INA-labeled 20-kDa T3 is composed of a 14-kDa protein backbone with an additional stretch of hydrophobic amino acids or a lipid tail. No evidence, however, for the presence of a lipid tail has been found yet; labeling studies of T3 with [^{3}H]palmitic acid were negative (not shown). However, a similar observation was made with rat and murine Thy-1 antigens, which on the basis of detergent-binding studies and sequence studies seem to contain a lipid tail [25]. If the INA-20-kDa T3 species contained a transmembrane segment consisting of hydrophobic amino acids, its location would probably be at the carboxyl terminus, since the preliminary N-terminal amino acid sequence of the combined 20-kDa T3 antigens was unambiguous (J. Borst and J. Coligan, unpublished). This suggests that the N terminus of the two 20-kDa T3 species is identical, although it cannot be excluded that the N terminus of one of the T3 forms is blocked. Clearly, more protein-chemical studies are needed to determine the structural relationship between the two T3 species.

The majority of the INA label was found in only one of the 20-kDa T3 species, which may indicate that this form contains a hydrophobic pocket, as discussed before. In Fig. 2, we showed that by cell surface radioiodination a T3 form was labeled, which had a pI identical to that of the T3 species which is susceptible to [^{125}I]INA labeling (position b). Yet, it was found invariably that practically all cell surface-radioiodinated material could be digested with Endo-F (Fig. 3, lane d). The fact that there are two T3 forms of slightly different molecular weights migrating at position 2 in IEF, could explain this finding. One of these forms would be glycosylated, the other not, according to the results presented in Fig. 4 and 5. The glycosylated 20-kDa T3-b form might then be predominantly labeled by cell surface radioiodination, whereas the unglycosylated T3-b form was labeled with the [^{125}I]INA compound. This finding is consistent with the idea that a substantial portion of the INA-labeled T3 species lies within the plasma membrane, or on its cytoplasmatic side. A similar case was reported for the IgE receptor on mast cells [19]. There, a 50-kDa glycoprotein subunit was found to be associated with a 30-kDa, [^{125}I]INA-labeled subunit. Thus, the INA-T3 species might be the anchor for the glycosylated 20-kDa T3 form and possibly also for the 25–28-kDa, 37-kDa and 44-kDa glycoproteins of the T3 complex. Perhaps these other glycoproteins are part of the T cell recognition structures through which the complex is involved in different T cell functions. An association between antigen recognition structures and those involved in T cell proliferation, for instance, could take place around the hydrophobic nucleus of the INA-T3 species. More studies of the molecular anatomy of the T3 complex are necessary to provide evidence for this proposition.

We are grateful to Drs. K. Hubbard, P. Robbins and P. Snow for helpful suggestions and to S. Lupul for assistance in typing the manuscript.

Received March 14, 1983.

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