SYNTHESIS AND PROCESSING OF MOLECULES
BEARING THYMUS LEUKEMIA ANTIGEN*

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The *Tla* locus, situated $\cong 1$ centimorgan from *H-2D*, specifies a number of antigens called TL (thymus leukemia)[1] that are expressed only on thymocytes at an early stage of their differentiation and on some leukemias (1, 2). This locus, which is thought to comprise a series of linked structural and regulatory genes (3), has several unique features. These cannot be reviewed here, but three points merit special attention in the context of the present report.

First, the TL product or products bear a physico-chemical resemblance to the K and D products of *H-2* and to at least one of the Qa molecules (4–10) specified by *Qa* genes linked to *Tla*, including association with β-2-microglobulin. Thus, TL typifies one family of glycoproteins specified by genes in and near the major histocompatibility complex.

Second, TL is lacking from the pro-thymocyte and becomes manifest when this precursor cell is induced to become a thymocyte; but TL is no longer expressed on the fully differentiated T lymphocytes that are derived from early thymocytes. (It is generally presumed that TL^+ thymocytes represent a normal stage of T-cell development, rather than an abortive side branch, but definitive proof is still lacking.)

Third, some strains of mice (TL^- strains) do not express TL on their thymocytes and yet do so on many of their leukemias, the set of TL antigens expressed on such leukemias being different from the TL set expressed by leukemias and thymocytes of TL^+ mice. Thus, in these two respects leukemogenesis is often associated with aberrations of TL expression.

For several reasons TL is an attractive model for studying the synthesis, regulation, and posttranscriptional elaboration of glycoprotein species selectively incorporated in the plasma membrane in the course of differentiation. As groundwork for such studies, we have investigated the synthesis and processing of TL molecules, whose expression might be expected to depend on appropriate association with β-2-microglobulin (11, 12) as well as a prescribed sequence of posttranslational glycosylation steps which is doubtless a general feature of the synthesis of cell-surface glycoproteins (13–17). High resolution gel electrophoresis was used to resolve different TL species obtained from lactoperoxidase-iodinated cells, in which the label is confined to molecules accessible

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[1] *Abbreviations used in this paper:* endo H, endo-β-N-acetylglucosaminidase H; NMS, normal mouse serum; PBS, Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+}; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulfate; TL, thymus-leukemia antigen.

at the cell surface, and from metabolically labeled cells, which also yield intracellular molecules bearing TL antigen. The various TL products were characterized according to the kinetics of metabolic labeling and to their carbohydrate chains. From these data it was possible to identify one intracellular glycoprotein which appears to be the presursor to cell-surface TL, and a second distinct glycoprotein which may be a product of the *Qa*-1 locus (18).

Materials and Methods

Animals and Cells.   All animals were bred and maintained in our colony at Memorial Sloan-Kettering Cancer Center. C57BL/6-*Tla^a* (B6-*Tla^a*) mice aged 3-6 wk were the source of all tissues from normal TL-positive mice. Age matched, B6-Lyt-2.1:3.1 and B6.K2 (19) mice served as TL-negative controls, the former for reasons of availability and the latter for reasons given in the text. The A strain leukemia ASL1 was passed at 10-d intervals in A/J mice. A tissue culture-adapted line of ASL1 cells, ASL1.1, was obtained from Dr. R. Hyman (Salk Institute).

Antisera.   The antiserum against TL.1,2,3,5 from (B6 × A-*Tla^b*)F₁ mice immunized with ASL1 cells is the standard TL-typing serum. A single pool was used throughout this work. There was no detectable difference between the molecular species precipitated by unabsorbed antiserum and species precipitated by antiserum that had been absorbed with AKR K36 leukemia cells to remove antiviral activity. Therefore, unabsorbed serum was used routinely. A pool of normal mouse serum (NMS), from strain 129 mice, was provided by Dr. L. Silver (Memorial Sloan-Kettering Cancer Center). Rabbit antiserum against rat β-2-microglobulin was a kind gift of Dr. L. Hood (California Institute of Technology).

Cell Culture and Metabolic Labeling.   Normal thymuses or spleens, or enlarged spleens from mice carrying the ASL1 tumor, were removed aseptically and minced in Dulbecco's phosphate-buffered saline (PBS). Clumps were allowed to settle, and the cells released into the supernate (>90% viable) were collected by low-speed centrifugation, washed with PBS, counted, and suspended at a density of 1-5 × 10⁷/ml for labeling. The labeling medium (prepared at this institute) was RPMI-1640 without methionine, supplemented with 50 μM 2-mercaptoethanol and 2% heat-inactivated fetal bovine serum. [³⁵S]Methionine, in sterile aqueous solution (New England Nuclear, Boston, Mass. translational grade), was added at 200-250 μCi/ml. For pulse labeling, the cells were initially suspended in the labeling medium without radioactive methionine and incubated at 37° for 10 min, to deplete intracellular methionine before addition of the label. The non-radioactive medium used for chase after a pulse label was RPMI-1640 with 20% heat-inactivated fetal bovine serum and 50 μM 2-mercaptoethanol.

To compare labeling with radioactive sugar and radioactive methionine, the cells were incubated for 12.5 h in medium with 8% serum, one-third of the normal methionine concentration, and either [³⁵S]methionine (200 μCi/ml), or [5,6-³H]fucose (100 μCi/ml) (New England Nuclear).

After labeling, cells were washed in RPMI-1640 without additives, and then either washed again in PBS before lysis or suspended in chase medium. The chase incubation was terminated by washing twice in ice-cold PBS. The cells were lysed in isotonic phospahte-buffered saline, pH 7.2, with 1 mM EDTA, 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl suflate (SDS), at a concentration of 2-10 × 10⁷ cells/ml. For cells harvested from the spleen, 1 mM phenylmethyl sulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, Mo.), an inhibitor of serine proteases, was added to the lysis buffer.

Lactoperoxidase-catalyzed Radioiodination.   Cells suspended at a concentration of ≅10⁸ per ml in PBS were iodinated by incubation with 100 μg of lactoperoxidase (Sigma Chemical Co.) and 2-2.5 mCi of Na ¹²⁵I (Amersham Corp., Arlington Heights, Ill.) at 22-25°C. The reaction was initiated by addition of 0.25 μmol of H₂O₂, with a second addition 5-10 min later. After the second 5- to 10-min interval, the reaction was terminated by washing the cells three times with 50 ml of ice-cold PBS containing 5 mM KI and 1 mM PMSF. The protease inhibitor, PMSF, could be omitted for thymocytes. Radioiodinated cells were then lysed as described for metabolically labeled cells.

Immune Precipitation.   Before use for immune precipitations, all lysates were precleared of material that precipitated nonspecifically. A 10% suspension of fixed, heat-killed Cowan I strain *Staphylococcus aureus* (Pansorbin, Calbiochem-Behring Corp., American Hoechst Corp., San

Diego, Calif.) was added to each lysate, at a concentration of ≅10 mg of bacterial cells per milliliter of lysate. After 15–60 min on ice, the lysates were clarified by centrifugation at 100,000 g for 90 min. The radioactivity incorporated into protein was measured by determining trichloroacetic acid (TCA)-precipitable, ethanol-insoluble radioactivity in samples of the lysates. Precleared lysates were stored at −70°C. After each thawing, the lysates were clarified in the Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.) before use.

For immune precipitation, samples of the lysates were incubated at 0°C for 10–20 h with a predetermined excess of anti-TL serum, usually 2 μl of a 1:10 dilution of the serum per 100 μl of lysate (10^7 cell eq). An equivalent amount of NMS was used in the controls. The immune complexes were collected in a 30- to 120-min incubation on ice with fixed S. aureus as described by Kessler (20). Precipitates were washed four times with PBS with 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS, then once with 10 mM Tris-HCl pH 7.5, 0.1% Nonidet P40. Bound IgG and antigen were usually released from the immune adsorbent by heating for 5 min at 80°C in sample buffer containing 2.4% SDS, 10% glycerol, 5% 2-mercaptoethanol, 65 mM Tris-HCl pH 6.8, and 0.002–0.01% bromophenol blue.

Slab Gel Electrophoresis. Samples were fractionated by electrophoresis on SDS-polyacrylamide gels, using a modification of the discontinuous buffer system of Laemmli (21). The separating gels were usually 10.5–11.5% polyacrylamide (acrylamide monomer: N,N-dimethyl bisacrylamide 30:0.8), in 0.75 M Tris-HCl, pH 8.8, and 0.2% SDS. The stacking gels were 3.6% polyacrylamide, 0.125 M Tris-HCl pH 6.8, and 0.2% SDS. Electrophoresis was at 35 mA constant current.

After electrophoresis, the gels were stained with Coomassie brilliant blue, destained with 10% acetic acid, and processed for fluorography as described by Bonner and Laskey (22). The dried gels were exposed to presensitized x-ray film (Kodak XR-2, Eastman Kodak Co., Rochester, N. Y.) at −70°C to obtain an approximately linear intensity response (23).

Treatment with Endo-β-N-acetylglucosaminidase H. Endo-β-N-acetylglucosaminidase H (endo H) was a kind gift of Dr. P. W. Robbins (Massachusetts Institute of Technology, Cambridge, Mass.). It was dissolved in 10 mM potassium phosphate buffer, pH 8.0, at a concentration of 100 μg/ml, and stored in aliquots at −70°C.

Samples were prepared for endo H digestion by immune precipitation as described above, except that the samples were released from the immunoadsorbent by heating in 1% SDS, 0.05 M Tris-HCl pH 6.8. The bacteria were removed by centrifugation, and aliquots of the supernate were diluted with an equal volume of 0.15 M Na citrate buffer, pH 5.5. The enzyme was then added, and the samples were incubated for 1–12 h at 30–31°C. After digestion, 1/10 vol of 20% SDS was added to each sample, the samples were heated to 80°C for 5 min, and the macromolecules were recovered by precipitation with 3–5 vol of ice-cold acetone.

Results

TL Molecules within the Cell and in the Plasma Membrane. TL antiserum, directed to the antigens TL.1,2,3,5, was used to detect molecules with TL specificity in lysates of B6-Tla^a (TL$^+$) thymocytes and of ASL1 leukemia cells of the A mouse strain. The Tla^a haplotype of the recombinant congenic strain B6-Tla^a was derived from the A strain. Thus, in both cells types the TL antigens, and any other antigens specified by genes in the differential segment of chromosome 17 (*Qa:Tla* region) bounded by the cross-over points, should be the products of the same block of genes.

Newly synthesized proteins were labeled biosynthetically by incubating the cells with [^{35}S]methionine for 3–4 h. Molecules accessible at the cell surface were labeled with ^{125}I by catalysis with lactoperoxidase. After each of these procedures, labeled molecules bearing TL were identified by reaction with TL antiserum, collection with fixed S. aureus, denaturation with SDS, and resolution by polyacrylamide gel electrophoresis (PAGE).

Fig. 1 shows several distinct TL species. In lysates of iodinated thymocytes and ASL1 cells, TL appeared in two forms, represented by a well-defined major band migrating with the mobility of a polypeptide of 46,000 daltons (46K TL) and a more
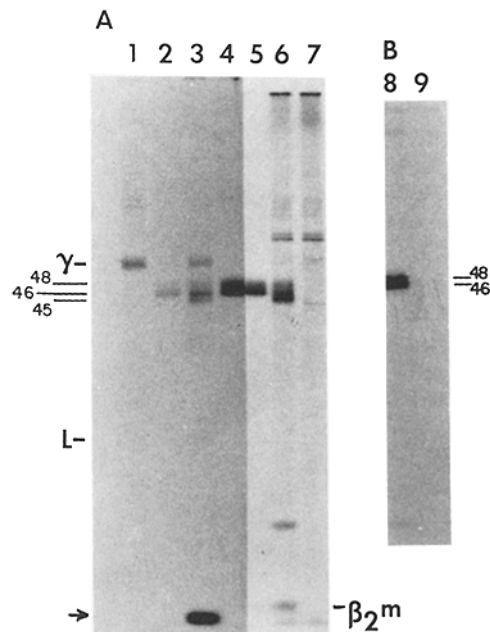
Fig. 1.   Surface and intracellular forms of TL.1,2,3,5 glycoproteins. (A) Lysates of ASL1 leukemia cells and B6-Tla^a thymocytes, labeled either by lactoperoxidase-catalysed radioiodination of intact cells or by metabolic incorporation of [^{35}S]methionine, were incubated with NMS or with TL antiserum. The precipitates were collected with S. aureus Cowan I strain, solubilized in sample buffer that contained SDS and 2-mercaptoethanol, and analyzed by electrophoresis on 11.5% polyacryl-amide gels. Metabolic labeling was for 3.5-4 h. The amount of TCA-precipitable radioactivity in each sample before precipitation is given in parentheses. Lane 1: [^{35}S]methionine-labeled ASL1 lysate, precipitated with NMS (5.1 × 10^6 cpm). Lane 2: anti-TL precipitate of ^{125}I-labeled ASL1 lysate (1 × 10^6 cpm). Lane 3: anti-TL precipitate of [^{35}S]methionine-labeled ASL1 lysate (7.5 × 10^6 cpm). Lanes 4 and 5: anti-TL precipitate, ^{125}I-labeled thymocyte lysate (2.7 × 10^5 cpm). Lane 6: anti-TL precipitate, [^{35}S]methionine-labeled thymocyte lysate (4.1 × 10^6 cpm). Lane 7: NMS precipitate, [^{35}S]methionine-labeled thymocyte lysate (4.1 × 10^6 cpm). Samples 1-4 and 5-7 were analyzed on separate gels. Fluorographic exposure was at −70°C for 14 d (lanes 1-4) or for 9 d (lanes 5-7). In this and subsequent figures, radioactive immunoglobulin (γ and L in figure) is synthesized by B splenocytes contaminating the ASL1 cells; these molecules bind to the S. aureus immunoadsorbent and copurify with the immune precipitate. The position of β-2-microglobulin in lanes 2 and 3 is indicated by an arrow. Apparent molecular weights of TL species (× 10^{-3} daltons) are indicated. (B) Lactoperoxidase-radioiodinated thymocytes from B6-Tla^a (lane 8) and B6.K2 (lane 9) mice were incubated with TL antiserum, and the precipitates were analyzed by electropho-resis on 10% polyacrylamide gels. The input radioactivity before precipitation was 3.8 × 10^5 cpm for lane 8 and 1.8 × 10^5 cpm for lane 9. Autoradiography was for 4 d.

diffuse component with an apparent mol wt (M$_r$) of 47,000-48,000 daltons (48K TL). Thymocytes usually gave a more prominent and better-defined 48K TL component than did ASL1 cells (Fig. 1, lanes 2, 4, and 5).

Expression of both 46K and 48K TL is controlled by genes closely linked to the Tla locus, because neither component was detected in the thymocytes of congenic recom-binant B6.K2 mice (Fig. 1, lanes 8 and 9). Although B6.K2 mice are indubitably TL-negative (genotype Tla^b) in immune cytotoxicity assays, it is not yet certain that they lack all or only some of the several Qa antigens recognized by the TL antiserum. Therefore more rigorous identification of the 46K and 48K molecules as TL must rest on further evidence, given later in this report.

Internal labeling with [^{35}S]methionine yielded both 46K and 48K TL and also a
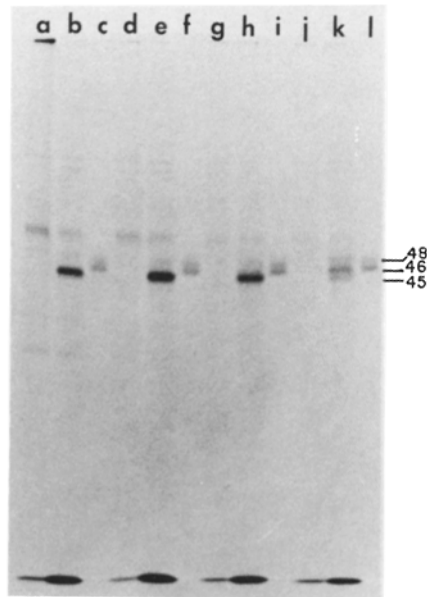
Fig. 2.   Pulse-chase analysis of TL from ASL1 cells. ASL1 cells were pulse-labeled for 15 min with [³⁵S]methionine, then incubated in complete medium without label for various periods of chase. Samples representing the lysate of 6×10^6 cells were precipitated with equivalent concentrations of normal mouse serum (lanes a, d, g, and j) or TL antiserum (lanes b, e, h, and k). TCA-precipitable radioactivity in each sample before immune precipitation is given in parentheses. Lanes a and b: 0-min chase (2,400,000 cpm). Lanes d and e: 20-min chase (2,100,000 cpm). Lanes g and h: 60-min chase (1,800,000 cpm). Lanes j and k: 180-min chase (1,500,000 cpm). Lanes c, f, i and l: radioiodinated thymocyte lysate precipitated with anti-TL serum. Fluorography was at −70°C for 14 d.

third species with an apparent M_r of 45,000 daltons (45K TL) (Fig. 1, lanes 3 and 6). This molecule was not accessible for iodination in intact cells; even prolonged autoradiographic exposure revealed no iodinated component with this mobility. Thus, unless 45K TL is a rare cell-surface molecule which lacks tyrosine, it is evidently an exclusively intracellular form of TL.

Both internally labeled and iodinated immune precipitates (visible on longer exposure) included a labeled subunit of low molecular weight identified as β-2-microglobulin by its comigration with the major product of precipitation with rabbit antiserum to rat β-2-microglobulin.

Precursor-Product Relationships of TL Molecular Species.   The relations of the 45K, 46K, and 48K varieties of TL to one another were investigated by pulse-chase labeling, which permits the study of newly formed proteins which have undergone posttranslational processing for selected intervals of time. Cells were labeled briefly with [³⁵S]-methionine (pulse) and then exposed during various time intervals to nonradioactive (cold) methionine (chase).

The results of these pulse-chase analyses in ASL1 cells and thymocytes are shown in Figs. 2 and 3, respectively. In each case, cells were labeled for 15 min and then chased with cold methionine for up to 3 h. The metabolically labeled TL molecules were compared in each case with iodinated cell-surface TL.

In both cell types, the only TL species labeled by a 15-min pulse was 45K, migrating distinctly faster than any iodinated cell-surface species (Fig. 2, lanes b and c; Fig. 3,
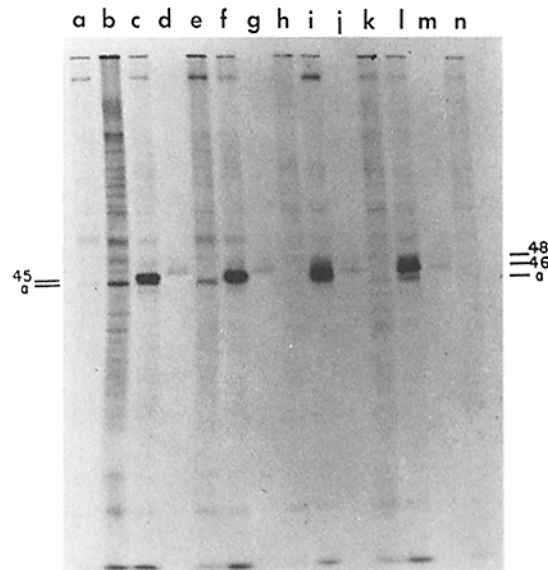
Fig. 3. Pulse-chase analysis of TL in B6-Tla^a and congenic TL-negative thymocytes. Thymocytes from B6-Tla^a(TL$^+$) or congenic B6-Lyt-2.1:3.1 (TL$^-$) mice pulse-labeled and chased as in Fig. 2. Samples of TL$^+$ represent 2.4 × 10^7 cell equivalents; samples of TL$^-$ represent 3.1 × 10^7 cell equivalents. Lane a: NMS precipitate from TL$^+$ cells, 20-min chase. Lane n: NMS precipitate from TL$^-$ cells, 60-min chase. All other samples precipitated with TL antiserum. Lane b, TL$^-$ cells, 0-min chase; lane c, TL$^+$ cells, 0-min chase. Lane e, TL$^-$ cells, 20-min chase; lane f, TL$^+$ cells, 20-min chase. Lane h, TL$^-$ cells, 60-min chase; lane i, TL$^+$ cells, 60-min chase. Lane k, TL$^-$ cells, 180-min chase; lane l, TL$^+$ cells, 180-min chase. Samples of TL$^+$ cells before precipitation contained 2.3–3.1 × 10^6 cpm in protein, depending upon the time of chase, and samples of TL$^-$ cells contained 2.9–4.4 × 10^6 cpm before precipitation. Lanes d, g, j, and m: anti-TL precipitate of radioiodinated ASL1 cell lysate. Fluorography was at −70°C for 7 d. The position of actin is marked (a).

lanes c and d). The cell-surface TL species 46K and 48K were only detectable after chase times of 60 min or more, and substantial accumulation of these molecules was only evident after a chase of 3 h (Fig. 2, lane k; Fig. 3, lane 1). By this time the amount of radioactivity in 45K TL had clearly declined. These labeling kinetics suggest that 45K TL may be a precursor which is converted to the cell-surface forms of posttranslational processing over a period of 1–3 h.

Conservation of radioactivity among all the TL species throughout the chase was poorer in ASL1 cells than in thymocytes (Fig. 2, lanes b, k; cf. Fig. 3, lanes c, 1). This may be due in part to a higher overall rate of protein turnover in the leukemia cells, or to selective degradation of surface TL by proteases contaminating the cell preparation. In the latter case, the rate of production of 46K and 48K may be underestimated (cf. Fig. 2, lane h, and Fig. 3, lane i).

In ASL1 cells, 48K TL could not be quantitated because of the low yield and a nonspecific background band in the same position. In thymocytes, however, the distinction of 48K TL from background was clear and its appearance was easier to follow. No evidence could be found for conversion of 46K TL to 48K TL.

The identification of a presumptive precursor (45K TL) to cell-surface TL raises the question whether thymocytes of Tla^b genotypes make 45K TL which fails to be converted to 46K/48K TL. This is an important point in view of our ignorance of how TL comes to be expressed on leukemia cells of these TL-negative mouse strains.

Therefore, TL-negative thymocytes were examined for intracellular TL after pulse labeling and various periods of chase (Fig. 3b, e, h, and k). No 45K TL was detectable in these lysates at any point. In occasional samples, nonspecific background precipitation was severe, and a band of actin was conspicuous at 43,000 daltons (Fig. 3b). This could be distinguished from authentic 45K TL, however, by a slight but definitive difference in mobility. Thus, it was possible to ascertain that TL⁻ thymocytes do not make any methionine-labeled product which is recognized by TL antiserum. Evidently TL⁻ thymocytes can do without TL altogether or substitute for it a gene product which does not cross-react immunologically.

High-mannose Carbohydrate in 45K TL. Molecules precipitable with TL antiserum have been characterized as glycoproteins (5). Therefore some of the differences between the forms of TL may reside in their carbohydrate portions.

Carbohydrate can be attached to proteins via an alkali-resistant bond to asparagine, or alternatively through an alkali-sensitive linkage to serine, threonine, or hydroxylysine (24). These later oligosaccharides, which are most common in secretory proteins, have structures and compositions very different from those of the asparagine-linked sugars, and the time of their addition to newly synthesized polypeptides is not known. The major histocompatibility antigens of humans and mice apparently lack this class of carbohydrate (25, 26), and no attempt was made to look for alkali-sensitive carbohydrate in TL in this study.

Glycoproteins may contain two types of asparagine-linked oligosaccharides: first, a high-mannose type, of simple composition, containing N-acetylglucosamine, occasionally glucose, and as many as ten mannose residues; and second, a low-mannose complex type, which contains only three or four mannose residues but may include fucose, galactose, and sialic acid (24, 27). It has been proposed that high-mannose oligosaccharides are initially added en bloc to nascent or newly-synthesized glycoproteins (27–33); the complex-type oligosaccharides are derived from these structures subsequently by removal of the glucose and excess mannose residues and addition of the other sugars. Therefore an intracellular precursor of a cell surface glycoprotein might be expected to contain the simple high-mannose type of side chain.

To test for such a high-mannose side chain directly, the various TL species were assayed for sensitivity to endo H (34). This enzyme has a well-documented specificity, cleaving off the high-mannose type of oligosaccharide side chain close to the asparagine linkage, but leaving the mannose-poor complex-type oligosaccharides untouched (27, 34). Alteration of the mobility of a protein on SDS-PAGE by treatment with endo H would be direct evidence of that protein's association with high-mannose oligosaccharide.

Different concentrations of endo H were used to digest the TL products of [³⁵S]-methionine-labeled ASL1 cells. Because endo H tolerates moderate concentrations of detergent, the TL-bearing molecules could first be purified from total cell lysates by immune precipitation with fixed *S. aureus* and released by heating in SDS. Endo H digestion products of pulse-labeled TL, TL labeled for 4 h and surface-labeled TL are compared in Fig. 4. Labeled immunoglobulin, synthesized by splenocytes contaminating the ASL1 population, served as an internal positive control for enzyme activity. Pulse-labeled 45K TL, as well as pulse-labeled immunoglobulin, was sensitive to endo H (Fig. 4, lanes 1–3), the 45K band being replaced primarily by a new species migrating faster than actin and with an M_r of ≅ 40,000 daltons. The reaction was partial with 1 μg/ml endo H and complete with 10 μg/ml. Even after continuous
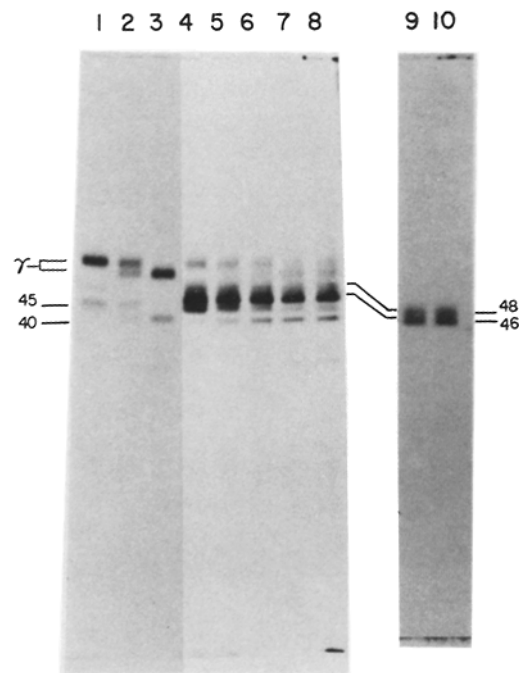
Fig. 4.   Endo H digestion of ASL1 TL. TL antigens were isolated by immune precipitation from lysates of ASL1 cells labeled for 15 min (lanes 1–3) or for 4 h (lanes 4–8) with [35S]methionine or surface-labeled by lactoperoxidase-catalyzed radioiodination (Lanes 9 and 10). The samples were divided into equal aliquots and digested with different amounts of endo H at 30°C for 75 min (lanes 1–8) or for 12 h (lanes 9 and 10). Lane 1: no endo H. Lane 2: 1 μg/ml endo H. Lane 3: 10 μg/ml endo H. Lane 4: no endo H. Lane 5: 0.3 μg/ml endo H. Lane 6: 1 μg/ml endo H. Lane 7: 3 μg/ml endo H. Lane 8: 10 μg/ml endo H. Lane 9: no endo H. Lane 10: 3 μg/ml endo H. The dye front, including the β-2-microglobulin band, was run off the gel.

labeling for 4 h the labeled molecules migrating as 45K TL were still sensitive to endo H (Fig. 4, lanes 4–8). In contrast, there was no loss or change in the mobility of 46K and 48K TL in these samples or in the iodinated samples. No labeled 40,000-dalton product was generated by digestion of surface-iodinated TL (Fig. 4, lanes 9, 10).

Two conclusions emerge from these data. First, the change in electrophoretic mobility of 45K TL cannot be ascribed to general protease contamination in the endo H preparation. Thus, the susceptibility of 45K TL to this enzyme is a result of the presence of carbohydrate of the high-mannose type. Secondly, any carbohydrate in the cell-surface forms, 46K and 48K TL, must have a different structure.

The pattern of susceptibility to endo H provides further insight into possible relationships between the TL species.

Migration of the major digestion product from 45K TL labeled for 4 h was identical to that of the 40K product of pulse-labeled 45K TL. Therefore, few if any intermediate processing steps, such as proteolytic cleavage or addition of serine- or threonine-linked sugars, are concealed by a compensating heterogeneity in the high-mannose carbohydrate of 45K TL. More probably, 45K TL is a unique and uniform species.

Furthermore, the resistance of 46K and 48K TL to endo H indicates that probably neither possesses any high-mannose chains. If they possess asparagine-linked carbohydrate, it must be exclusively of the complex type. The all or none behavior of 45K and 46K TL may indicate that there is only a single carbohydrate chain per
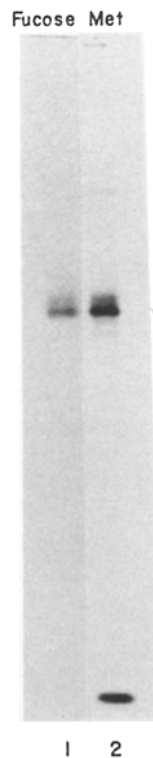
Fucose Met

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Fig. 5.   Metabolic sugar labeling of ASL1 TL. ASL1 cells were labeled for 12.5 h with [^{35}S]-methionine or [^{3}H]fucose. Samples were precipitated with TL antiserum. Lane 1: fucose-labeled lysate. Lane 2: methionine-labeled lysate.

glycoprotein molecule, or alternatively that different carbohydrate chains attached to the polypeptide are processed for the most part synchronously.

Carbohydrate of Cell Surface TL.   Further evidence for complex-type oligosaccharide in cell surface TL was provided by direct metabolic sugar labeling. As shown in Fig. 5, both 46K and 48K TL were labeled after a 12.5-h incubation with [5,6-^{3}H]fucose. This sugar is not interconverted with other monosaccharides before it is incorporated into glycoproteins (35); therefore both cell-surface TL species are fucosyl glycoproteins. Addition of fucose is a late event in the processing of oligosaccharide chains (36). Its presence in 46K and 48K TL thus indicates that they are extensively processed glycoproteins with complex-type oligosaccharides (24).

In other experiments (data not shown), 46K and 48K TL displayed more acidic isoelectric points than 45K TL, suggesting the presence of sialic acid in their carbohydrate chains.

Tissue Specificity of TL Species.   The prototypic TL antigens detected by immune cytotoxicity are restricted to the thymus, being found in the periphery only in mice with TL^{+} leukemias. To identify the 45K, 46K, and 48K molecules as TL more accurately, it was important therefore to determine whether synthesis of these glyco-proteins is similarly restricted to thymocytes. Spleen cells from B6-*Tla^{a}* and congenic TL-negative mice were labeled with [^{35}S]methionine for 30 min and for 3 h, and the lysates were precipitated with TL antiserum as shown in Fig. 6. Two discrete species
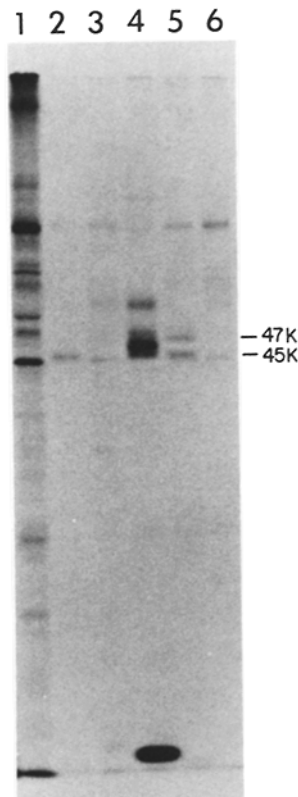
Fig. 6. An antigen in spleen linked to the *Tla* locus. Spleen cells from normal B6-*Tla*^{a}(TL$^+$) and B6-Lyt 2.1, 3.1 (TL$^-$) mice were labeled with [^{35}S]methionine for 30 min or 3 h. The lysates were then subjected to immune precipitation with TL antiserum, except for the sample shown in lane 1. The input radioactivity in each sample before precipitation is given in parenthesis. Lane 1: 3-h labeled TL$^+$ spleen, control precipitate with NMS (4,000,000 cpm). Lane 2: 30-min labeled TL$^+$ spleen (530,000 cpm). Lane 3: 30-min labeled TL$^-$ spleen (1,160,000 cpm). Lane 4: 4-h labeled ASL1 lysate (same as in Fig. 4, lane 4; 5,000,000 cpm). Lane 5: 3-h labeled TL$^+$ spleen (4,000,000 cpm). Lane 6: 3-h labeled TL$^-$ spleen (6,000,000 cpm).

were found in the immune precipitates (Fig. 6, lane 5). Neither was synthesized by splenocytes from congenic TL-negative mice (Fig. 6, lanes 3 and 6), and neither was precipitated by normal mouse serum (Fig. 6, lane 1). One of these species, with an apparent M_r of 44,000–45,000 daltons, was labeled within 30 minutes (Fig. 6, lane 2). The second, migrating like a polypeptide of 47,000 daltons, became radioactive only after a longer labeling period (Fig. 6, lane 5), and might be a derivative of the first product. Precipitates from surface-iodinated spleen cells only displayed the 47,000-dalton component (data not shown).

Thus, the peripheral lymphoid cells of TL-positive mice synthesize polypeptides that are recognized by a TL antiserum and are expressed under the control of a gene or genes linked to *Tla*. This result is compatible with the fact that TL antiserum recognizes Qa allotypes expressed on peripheral lymphocytes.

Of the two splenic molecular species, the 45,000-dalton molecule resembled 45K TL in both labeling kinetics and mobility on SDS-PAGE. Although Qa antigens are serologically distinct from TL antigens (18), it is not known whether the polypeptide
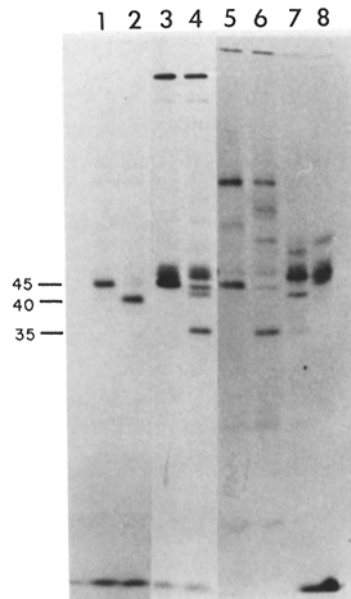
Fig. 7. Comparative endo H digestion of antigens recognized by anti-TL serum. Antigens reactive with TL antiserum were isolated by immune precipitation from lysates of ASL1.1 tissue culture cells, B6-Tla^a thymus, B6-Tla^a spleen, and ASL1 cells propagated in vivo. In each case the cells were labeled with [^{35}S]methionine for 3–4 h, except that the ASL1.1 cells were labeled for 0.5 h. The samples were divided in half and incubated with or without 3.3–10 μg/ml of endo H for 1–3 h at 31°C. Lane 1: ASL1.1, no endo H. Lane 2: ASL1.1, after endo H. Lane 3: thymus, no endo H. Lane 4: thymus, after endo H. Lane 5: spleen, no endo H. Lane 6: spleen, after endo H. Lane 7: ASL1 from spleen, after endo H. Lane 8: ASL1 from spleen, no endo H. The figure is a composite from 3 gels, 1–2, 3–4, and 5–8, run on different occasions.

backbones of these glycoproteins differ. Therefore it was important to determine whether the pulse-labeled splenic product was identical with the 45K intracellular form of TL.

If the two molecules were identical, both should migrate identically after digestion with endo H. To test this, anti-TL immune precipitates from ASL1, from thymocytes, and from splenocytes, were digested with endo H and compared as shown in Fig. 7. As before, 45K TL from thymocytes and from ASL1 was characteristically sensitive to endo H (Fig. 7, lanes 2, 4, and 7). The 45,000-dalton splenocyte molecule was also susceptible to endo H (Fig. 7, lane 6), but after digestion there was no sign of the characteristic 40,000 dalton TL product (cf. lanes 6 and 7). Instead there appeared a new digestion product with an M_r of ≅35,000 daltons. If all asparagine-linked sugar in the newly made glycoproteins is sensitive to endo H (27), this experiment defines two differences between 45K TL and the splenocyte glycoprotein. First, the splenocyte polypeptide is apparently ≅5,000 daltons smaller; second, it must contain more high mannose carbohydrate to give the apparent mol wt of 45,000 daltons. If the length of each high-mannose chain is the same, the splenocyte molecule must possess additional sites of glycosylation. Therefore the 45,000 dalton splenocyte glycoprotein is not 45K TL, but most likely another gene product.

Lysates of ASL1 cells from the spleens of A/J mice contained small amounts of the 35,000 dalton endo H digestion product, as well as the 40,000 dalton product

characteristic of TL (Fig. 7, lane 7; also see Fig. 4). However, a tissue culture line of ASL1 cells made only the 40,000 dalton product (Fig. 7, lane 2). Therefore, the 35,000 dalton product in lysates of ASL1 cells propagated in vivo was probably made by contaminating splenocytes, and not by the TL-positive leukemia cells.

Both products were prominent in the thymocyte sample after endo H treatment (Fig. 7, lane 4). Clearly the two gene products may be synthesized at similar rates in unfractionated thymocytes, although in other experiments the 40,000 dalton product predominates (E. Rothenberg, unpublished observations). The 45K pulse-labeled band from thymocytes is therefore composed of at least two glycosylated early molecules. The 47,000-dalton splenocyte molecular species may also be expected to contribute to the heterogeneity of the 46,000–48,000 dalton species recognized by TL antiserum which appear in a chase.

Thus, at least two distinct products were defined, both of them related to genes in the $Qa:Tla$ region. One, with a 40,000 dalton endo H-resistant core, occurs in thymocytes and ASL1 leukemia cells, but not in splenocytes. By tissue distribution, genetics, and serology, this glycoprotein can be equated with TL. A second gene product has an endo H-resistant core of $\cong 35,000$ daltons and is synthesized by splenocytes and by thymocytes. Preliminary data suggest that the two gene products are synthesized by different sets of thymocytes. This is of much interest, because TL is thought to be the hallmark of a subterminal cell set, the major thymocyte population, that yields terminally differentiated $TL^-:Qa^+$ cells represented in the minor thymocyte population and in the periphery.

Discussion

We have identified a group of related glycoproteins that appear to represent precursor and mature forms of the TL molecules in thymocytes and ASL1 leukemia cells. These are hardly major methionine-labeled constituents of the cells, for to obtain a gel band representing at most 200 cpm, it was necessary to use saturating amounts of antibody to precipitate antigen from a sample of cell lysate yielding $\cong 1 \times 10^6$ cpm. It was possible to follow the synthesis and processing of these molecules largely because of the high titer and fine specificity of the antiserum, which allow the background of nonspecific precipitation to be minimized.

According to evidence presented here, the processing scheme for these glycoproteins seems to follow the pattern established for a number of other viral and cellular glycoproteins (27–33). The first labeled product with TL antigenicity is probably a polypeptide of 40,000 daltons, containing enough asparagine-linked high-mannose oligosaccharide to make it migrate in SDS gels like a protein of 45,000 daltons. This initial species, 45K TL, is apparently converted to a slower-migrating complex of species, 46K and 48K TL, by a series of steps which must include come processing of the carbohydrate chains. Mannose residues must be removed, to make the oligosaccharide resistant to endo H, and fucose must be added, probably along with galactose and sialic acid. Whether, in addition, the polypeptide is subjected to proteolytic cleavage or further glycosylation at other sites is not known. In any case conversion to the 46K TL form seems to be a prerequisite for display of the TL glycoprotein on the cell surface.

All these processing steps are potential control points, and so it was important to examine phenotypically TL-negative tissues, such as spleens of genotypically TL-positive mice, and genotypically TL-negative tissues, ie, thymuses of genetically TL-

negative mice. In neither case was 45K TL, the intracellular precursor species, detectable. Therefore, in these two cases, a block in the conversion of 45K to surface TL is not the cause of the TL-negative phenotype. However, in the interpretation of these results, it must be emphasized that every molecule successfully precipitated here by the anti-TL serum is glycosylated. There is no evidence as yet that the stripped polypeptide backbone of the molecule or an aberrantly glycosylated TL glycoprotein can be recognized by TL antiserum. Therefore it is possible that a defect in initial glycosylation of the nascent TL polypeptide could make an otherwise-normal TL gene product inaccessible to our reagents. Further work will be needed to determine whether any of these post-translational processing events is used physiologically to regulate the expression of TL.

Finally, it was possible to identify a non-TL product recognized by the TL antiserum in thymus and spleen. Using the glycosidase endo H to dissect the newly-synthesized glycoprotein molecules, we were able to show that the antigen recognized in spleen is probably a separate gene product and not an altered form of the TL glycoprotein. This gene product is specific for the Tla^a haplotype, and may correspond to Qa-1 (18).

Summary

Thymus-leukemia (TL) antigens are expressed in murine lymphocytes under strict developmental regulation. To elucidate the molecular basis of TL expression, we have identified the molecular species that react with TL antiserum. At least three species can be resolved by metabolic radiolabeling of thymocytes and ASL1 leukemia cells, lysis, immune precipitation, and sodium dodecyl sulfate-polyacrylamide. After a brief incubation with [35S]methionine, the only radioactive molecule recognized by TL antiserum is a homogeneous species with an apparent M_r of 45,000 daltons. This molecule, 45K TL, includes high-mannose-type carbohydrate attached to a 45,000 dalton glycosidase-resistant backbone. In this form, 45K, it is never exposed on the cell surface. If pulse-labeled cells are further incubated with nonradioactive methionine before lysis, however, radioactivity disappears from the 45K TL species and appears in the slower migrating species 46K and 48K TL. Thus, 46K and 48K appear to represent products generated from the 45K TL precursor by posttranslational modification. These TL forms are displayed on the cell surface; they lack high-mannose carbohydrate but evidently include acidic complex-type carbohydrate. Normal thymocytes from $Qa:Tla$-negative mice lack not only the surface forms of TL but also the intracellular 45K TL form.

Peripheral lymphoid cells of $Qa:Tla$-positive mice synthesize none of these TL species. But the TL antiserum, which contains Qa antibody, recognizes a distinct gene product in spleen and thymus of $Qa:Tla$-positive mice. In its pulse-labeled form, this molecule, which may represent Qa-1, has an apparent M_r of 44,000 daltons, and consists of a glycosidase-resistant polypeptide core of only 35,000 daltons linked to more high mannose carbohydrate than 45K TL.

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