HUMAN HOMOLOGUE OF MURINE T200 GLYCOPROTEIN*

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One approach to understanding the molecular basis of the cellular interactions that take place during the immune response is to identify and characterize the cell-surface glycoproteins of the cell types involved to identify molecules that may play a role in these processes. In man, cell-surface glycoproteins of thymus-dependent (T) and thymus-independent (B) lymphocytes and lymphoblastoid cell lines have previously been analyzed by a combination of various surface-labeling techniques, immunoprecipitation with conventional xenoantisera, and analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)[1] (1–3). Comparison of the surface proteins of HSB2, a human T leukemic cell line, and CCRF-SB, a normal B cell line from the same patient, by these methods, revealed characteristic differences in their high molecular weight surface molecules: the B cell line expressed a molecule with an apparent molecular weight of ~220,000, whereas HSB2 cells expressed a molecule with an apparent molecular weight of 200,000 (1). Similar differences in the high molecular weight glycoproteins of normal human T and B lymphocytes have also been reported (2, 3).

Recently, the monoclonal antibody technique (4) has greatly facilitated the analysis of surface molecules of human lymphoid cells (5). With this technology, we report here data that demonstrate the differences between the high molecular weight surface molecules of human T and B cells can be accounted by the presence of structurally distinct but antigenically related glycoproteins on their cell surface. Chemical characterization, including two-dimensional peptide mapping, shows that these glycoproteins are the human homologues of murine T200 glycoprotein (6, 7).

Materials and Methods

Cell Lines and Tissues. All cultured human cell lines, including CCRF-HSB2 (8), a T cell line established from a patient with a T cell-acute lymphocytic leukemia; CCRF-SB (9), a normal B cell line from the same patient as CCRF-HSB2; and CCRF-CEM (10), another T cell-leukemic cell line, were grown in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5–10% fetal calf serum. Mouse T lymphoma cell lines, ASL1 and BW5147 (11), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum. Homogenates from tissues obtained at postmortem within a few hours of death and stored at −70°C were prepared at 4°C with an Omni mixer (Ivan Sorvall, Inc., Norwalk, Conn.) followed by filtration through nylon gauze and extensive washing with 0.15 M NaCl-0.01 M phosphate buffer (pH 7.2). Peripheral blood lymphocytes were isolated from defibrinated blood by Ficoll-Hypaque gradient centrifugation (12). Granulocytes were isolated from peripheral blood by mixing 40 ml of defibrinated blood with 20 ml of 5% (wt/vol) dextran

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[1] *Abbreviations used in this paper:* H-BSS, Hepes-buffered balanced salt solution; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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(clinical grade; Sigma Chemical Co., St. Louis, Mo.) in Hepes-buffered balanced salt solution (H-BSS). After a 30-min incubation at 37°C, the leukocyte-rich supernate was removed, and a cell pellet was obtained by centrifugation. The cells were washed twice with H-BSS, then layered over Ficoll-Hypaque. After centrifugation, the granulocyte-rich cell pellet obtained was treated with Tris-buffered NH_4Cl to lyse contaminating erythrocytes (13).

Immunological Reagents and Procedures. Monoclonal antibody T29/33 was obtained from a fusion of spleen cells from BALB/c mice immunized against the human cell line, CCRF-CEM, with the nonproducer myeloma cell line, S194/5.XXO.BU.1 by standard procedures (4, 7). Immunoprecipitation studies were carried out with ascitic fluid from tumor-bearing mice. The procedures used were essentially as described in detail previously (14). Trace antibody binding, quantitative immunofluorescence with the fluorescence-activated cell analyzer, and absorption assays were done as previously described, except for minor modifications (7, 15). For these experiments, monoclonal T29/33 antibody from culture supernate was used. For immunofluorescence studies of frozen tissue sections, portions of tissue were obtained from surgery or postmortem and rapidly frozen. Cryostat-frozen sections, 5-μm thick, were collected on acid-cleaned microscope slides, air-dried for 30 min, and rehydrated for 10 min in Dulbecco's phosphate-buffered saline (PBS). The sections were then flooded with the undiluted tissue culture supernate that contained the monoclonal antibodies. After a 1-h incubation at room temperature, they were given three rinses of 10 min each with PBS and incubated for another hour with fluorescein isothiocyanate-conjugated goat anti-mouse IgG at a 1:100 dilution (Antibodies Inc., Davis, Calif.). After another 10-min rinse with PBS, they were cover-slipped with Gelvatol (Monsanto Co., St. Louis, Mo.) and examined and photographed with a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York) equipped with an HBO 50-W light source and BG3 excitation filter. Sections incubated with supernates that contained unrelated antibodies or PBS were used as negative controls.

Biochemical Procedures. Lactoperoxidase-catalyzed iodination, metabolic labeling with L-[^{35}S] methionine, and proteolysis studies were carried out as described previously (14). Peptide mapping analysis was also performed as described previously for studies of murine T200 glycoprotein (14), except that labeled protein *Staphylococcus aureus* immunoprecipitates were reduced and carboxymethylated before preparative SDS-PAGE. This modification improves the recovery of labeled protein and gives identical maps to the procedure used previously.

Results

Identification of the Human Homologue of Murine T200 Glycoprotein. Previous studies had shown that human T and B lymphoid cells exhibited differences in their high molecular weight surface glycoproteins detected by surface-labeling techniques and immunoprecipitation (1–3). These observations are similar to those obtained in earlier studies of the cell-surface glycoproteins of mouse lymphoid cells (6, 7). In the mouse, we now know that the differences in the high molecular weight proteins of T and B lymphocytes detected by analysis on SDS-PAGE are attributable to the presence of structural variants of the glycoprotein referred to as T200 (7, 15). We therefore investigated whether the differences in high molecular weight glycoproteins of human T and B cells could be accounted for by a human homologue of murine T200.

A monoclonal antibody against a possible candidate for the human homologue of T200 glycoprotein was obtained from a fusion of mouse spleen cells immunized with the human T cell line, CCRF-CEM, with the mouse myeloma, S194/5.XXO.BU.1 as described in Materials and Methods. In preliminary experiments, by trace antibody binding and/or indirect immunofluorescence, this antibody, designated T29/33, reacted with 41 of 45 human hematopoietic cell lines tested and with >95% of peripheral blood mononuclear cells and granulocytes. A labeled molecule with an apparent molecular weight of ~200,000, which, on the basis of metabolic-labeling studies with mannose and galactose is a glycoprotein, was precipitated by T29/33

monoclonal antibody from a lysate of iodinated CCRF-CEM cells (data not shown). When similar iodination experiments were carried out with HSB2 and CCRF-SB cells, as shown in Fig. 1, the antibody precipitated a glycoprotein with an apparent molecular weight of ~220,000 from the B cell line, CCRF-SB, and a glycoprotein with an apparent molecular weight of 200,000 from the T cell-leukemic cell line, HSB2. Thus, the differences in the high molecular weight glycoproteins of CCRF-SB and HSB2 cells previously detected by conventional xenoantisera can be explained by these two antigenically related glycoproteins.

Comparison of [^{35}S]Methionine Tryptic Peptides from Murine T200 and the Glycoprotein of Human Lymphoid Cells Defined by T29/33 Monoclonal Antibody. The results reported in the previous section suggested that T29/33 monoclonal antibody may indeed react with the human homologue of murine T200 glycoprotein. To investigate this possibility, murine T200 glycoprotein and the putative human homologue were labeled with [^{35}S]methionine, isolated by immunoprecipitation and SDS-PAGE, and their tryptic peptides compared by two-dimensional tryptic peptide analysis. Two murine T cell-leukemic cell lines, ASL1 and BW5147, were used as a source of T200 glycoprotein; the human glycoprotein was isolated from HSB2 cells or another human T cell-leukemic cell line, CCRF-CEM. No differences were detected between the tryptic peptides from the glycoproteins isolated from the two different cell lines from each species. As shown in Fig. 2, the labeled tryptic peptides obtained from T200 glycoprotein were as previously reported (14). It is clear from a comparison with those of the human glycoprotein that both molecules share a substantial number of methionine-containing tryptic peptides. This was confirmed by analysis of a mixture of the peptides of both molecules. We estimate that 17 labeled peptides are shared by the mouse and human glycoproteins, 15 peptides are unique to murine T200, and 13 peptides are unique to the human homologue.

Tissue Distribution of Human T200 Glycoprotein Homologue. In the mouse, T200 glycoprotein appears to be restricted to cells of the hematopoietic series. Within the hematopoietic system, however, the glycoprotein is broadly distributed, being present on T and B lymphocytes, myeloid cells and their precursors, and immature erythroid cells. We have investigated the tissue distribution of the human T200 homologue by trace-antibody binding, quantitative absorption, and quantitative immunofluorescence with the fluorescence-activated cell analyzer and immunofluorescence microscopy of frozen sections. As described earlier, the glycoprotein is found in various amounts on the majority of human hematopoietic cell lines and on >95% of peripheral blood leukocytes. A similar broad distribution was found in other lymphoid and hematopoietic tissues examined, which included spleen, thymus, bone marrow, and tonsil. As shown in Fig. 3, immunofluorescence microscopy of frozen sections of tonsil and spleen revealed strong and relatively uniform staining of all the lymphoid cells present in both organs.

The distribution of human T200 glycoprotein on nonhematopoietic cells was investigated by quantitative absorption with tissue homogenates and intact cells. As shown in Table I, the glycoprotein was not detected on brain, liver, kidney, or erythrocytes. As found previously for other monoclonal antibodies (7), the absorption test was relatively insensitive, and the lower limit of detection was ~10% of the amount of the glycoprotein expressed on the human spleen cells. Except for the occasional lymphoid cell, however, we did not detect fluorescent staining of any cell

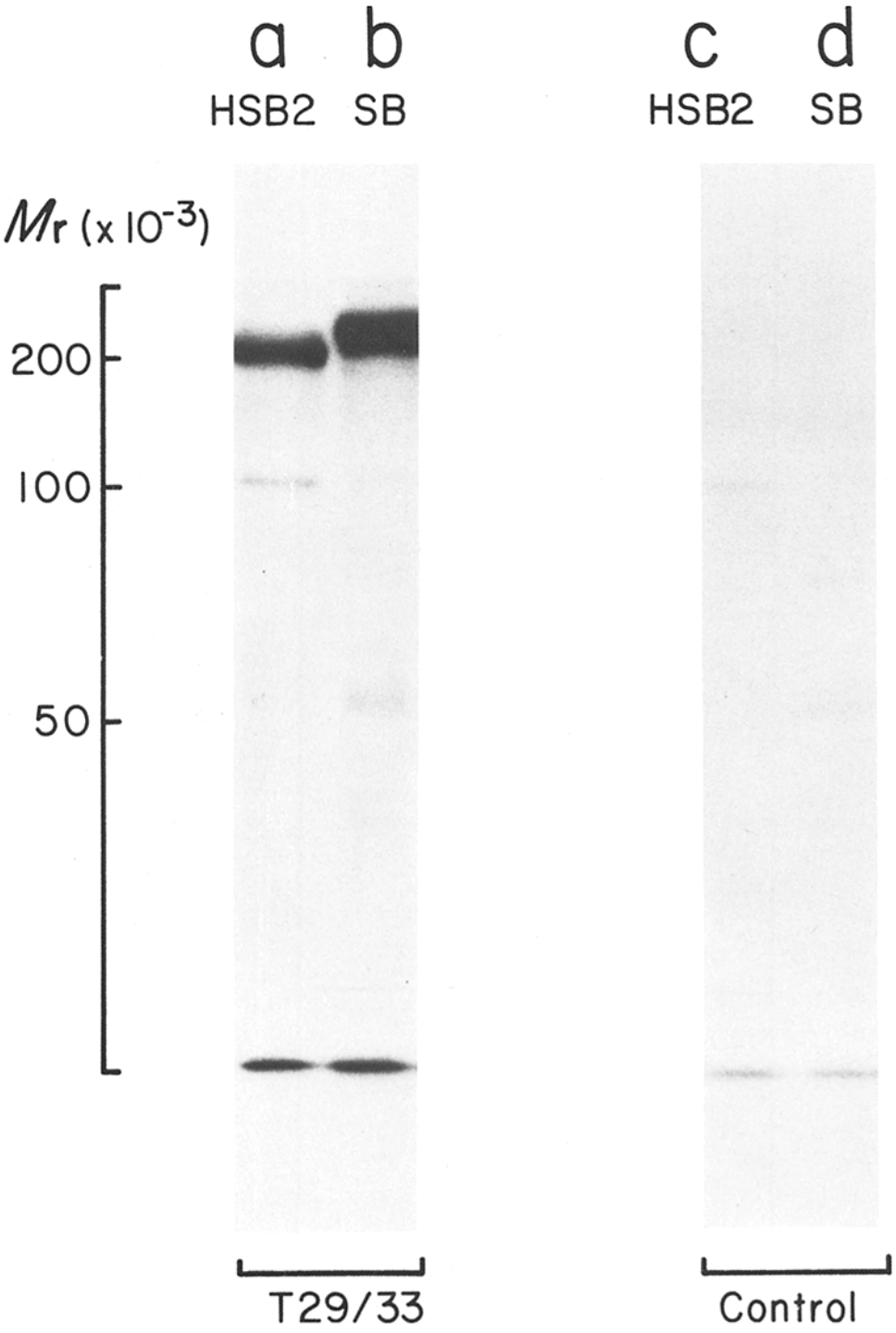

FIG. 1. Immunoprecipitation of T29/33 glycoprotein from ^{125}I-labeled CCRF-HSB2 and CCRF-SB cells. The autoradiograph of a 10% SDS-polyacrylamide gel of immunoprecipitates prepared from lysates of CCRF-HSB2 (tracks a and c) and CCRF-SB cells (tracks b and d) is shown. Antibodies: a and b, T29/33 monoclonal antibody plus goat anti-mouse IgG; c and d, goat anti-mouse IgG alone as control. M_r, apparent molecular weight.

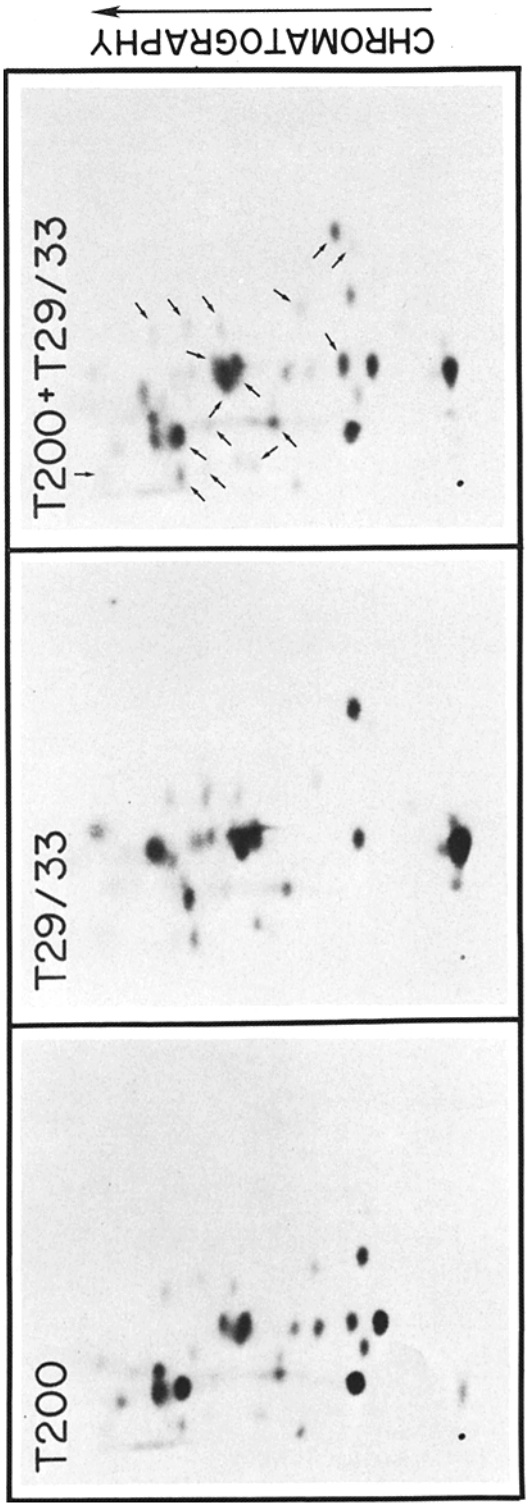

FIG. 2. Comparison of the tryptic peptides of T200 glycoprotein from murine ASL1 cells and T29/33 glycoprotein from CCRF-HSB2 cells metabolically labeled with L-[^{35}S]methionine. Tryptic peptides from the glycoproteins isolated by immunoprecipitation and SDS-PAGE, as described in Materials and Methods, were prepared and analyzed on thin-layer cellulose plates by electrophoresis in the first dimension and chromatography in the second dimension. Shown are autoradiographs of T200 glycoprotein (8-d exposure), T29/33 glycoprotein (14-d exposure), and a mixture of the two glycoproteins (14-d exposure) that contained approximately equal radioactivity from each. The arrows indicate common peptides between the mouse and human homologues.

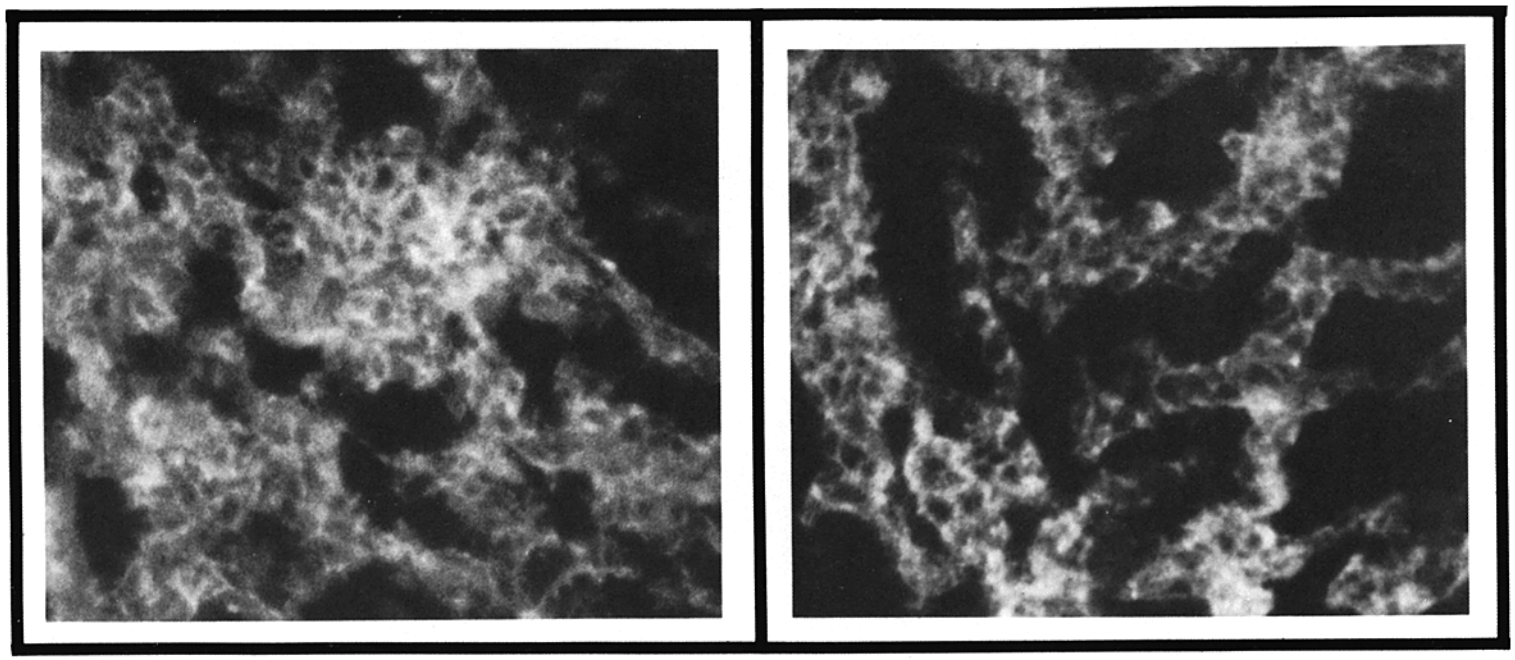

FIG. 3. Indirect immunofluorescence staining of human spleen and tonsil by T29/33 monoclonal antibody. Cryostat sections of spleen (a) and tonsil (b) were prepared and stained as described in Materials and Methods. Note the relatively uniform and bright staining of all lymphoid cells. No fluorescence was obtained if T29/33 antibody was omitted or other irrelevant monoclonal antibodies substituted.

TABLE I

Tissue Distribution of Human T200 Glycoprotein

Tissue	Relative amount of glycoprotein	Immunofluorescence
Spleen	1.0	+++
Kidney	<0.12	-*
Liver	<0.12	-
Brain	<0.12	-
Erythrocytes	<0.04	-
Lung	ND‡	-
Adrenal	ND	-
Myocardium	ND	-
Prostate	ND	-
Breast fibroadenoma	ND	-
Tonsil	ND	+++
RAJI Burkitt's lymphoma cell line	0.26	ND
CCRF-CEM T cell-leukemic cell line	0.33	ND

The results of quantitative absorption experiments and indirect immunofluorescence studies of cryostat tissue sections are shown. For quantitative absorption, equal volumes of T29/33 antibody at a dilution that was limiting in the trace antibody-binding assay were incubated for 1 h at 4°C with various amounts of cells or tissue homogenates and vortexed at 15-min intervals. The mixtures were centrifuged, and the supernates were assayed in duplicate for residual binding to CCRF-HSB2 cells. The table gives the relative absorption calculated from the packed volumes of cells or homogenate required for 50% absorption of T29/33 antibody. For immunofluorescence, cryostat sections were prepared and stained as described in Materials and Methods and Fig. 3. It should be noted that in many tissues occasional lymphocytes were found that stained strongly with T29/33 monoclonal antibody. All tissues were stained with an anti-β_2-microglobulin monoclonal antibody.

* Negative except for lymphoid cells.

‡ Not determined.

type in frozen sections of a variety of nonhematopoietic tissues, which suggests that the glycoprotein is only expressed in significant quantities on hematopoietic cells (Table I).

Proteolysis of Human T200 Glycoprotein. The structural relationship of mouse and human T200 glycoproteins was examined further by proteolysis experiments. We have previously shown that after solubilization in Nonidet P-40, murine T200 glycoprotein is rapidly cleaved by trypsin and several other proteases to give a protease-resistant fragment with an apparent molecular weight of ~100,000 (14). A similar fragment can be obtained in low yield by treating intact cells with proteases.

The results of trypsin treatment of the human T200 homologue on intact HSB2 cells or after solubilization in Nonidet P-40 are shown in Fig. 4. Treatment of iodinated HSB2 cell lysates with trypsin for 20 min gave two labeled fragments with apparent molecular weights of ~110,000 and 70,000 in good yield (Fig. 4). These fragments were resistant to further degradation by trypsin because similar results were obtained with a 60-min incubation (data not shown). Treatment of intact cells with trypsin, in contrast to murine T200 glycoprotein, also led to the quantitative cleavage of the human homologue. Under these conditions, only the labeled 70,000-mol wt fragment was obtained. Unlike the proteolytic fragment of murine T200 glycoprotein

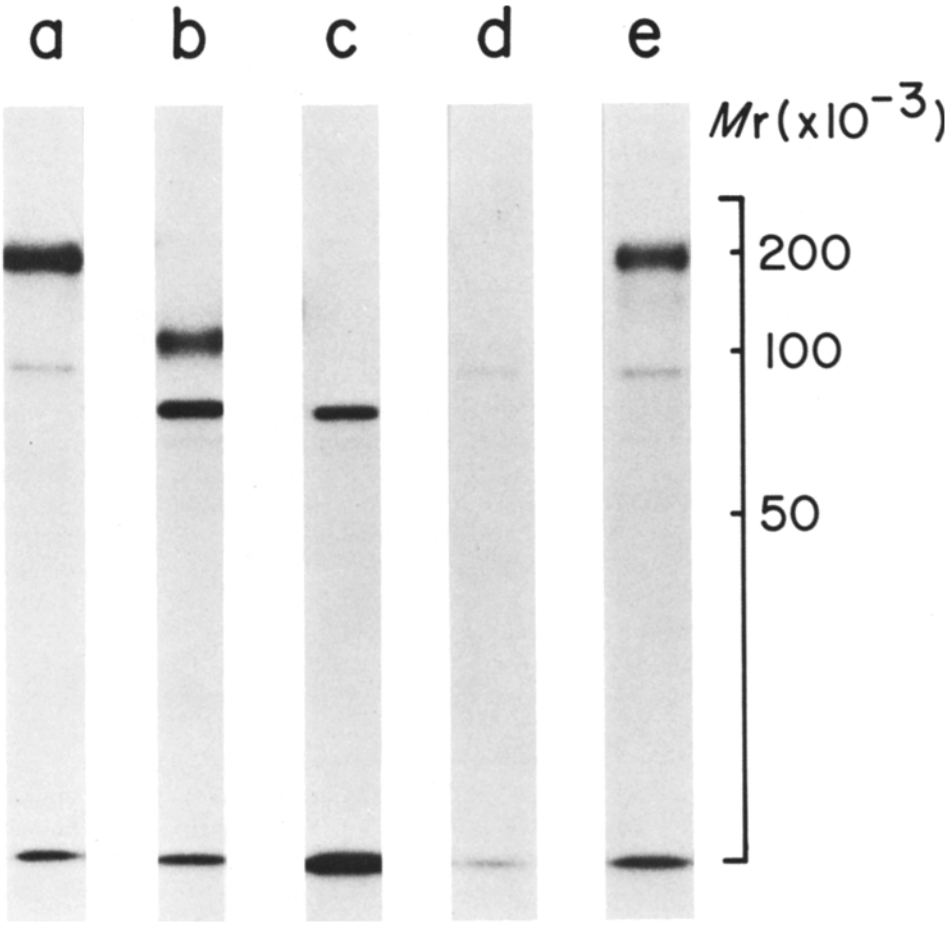

FIG. 4. Trypsin digestion of ^{125}I-labeled human T200 homologue. HSB2 cells were labeled by lactoperoxidase-catalyzed iodination as described in Materials and Methods. Labeled cells (1×10^7 cells in 1.0 ml of 0.15 M NaCl-0.01 M phosphate buffer [pH 7.2]) were either directly treated with trypsin (80 μg/ml for 10 min at 23°C) or first solubilized at the same cell concentration in 0.15 M NaCl-0.01 M phosphate buffer that contained 1% Nonidet P-40 and then digested with trypsin (50 μg/ml for 20 min at 23°C). Proteolysis was terminated by addition of phenylmethylsulfonyl fluoride and ovomucoid trypsin inhibitor. After immunoprecipitation, samples were analyzed by electrophoresis in a 10% SDS-polyacrylamide gel. Shown is the autoradiograph of gel tracks: (a) T29/33 glycoprotein from untreated cells, (b) T29/33 glycoprotein treated with trypsin after solubilization, (c) T29/33 glycoprotein isolated from the incubation supernate after trypsin digestion of intact cells, (d) immunoprecipitation control in which T29/33 antibody was omitted, and (e) proteolysis inhibition control in which protease inhibitors were added to a sample of cell lysate before addition of trypsin. (Track e is identical to inhibition control of T29/33 on intact cells, which is not shown.) M_r, apparent molecular weight.

that remains associated with the cell pellet after trypsinization (14), the tryptic cleavage product of the human glycoprotein is released almost quantitatively from the cell surface (Fig. 4).

Discussion

The results reported in this paper demonstrate that the differences in high molecular weight glycoproteins of human T and B cells detected by surface labeling and analysis

by SDS-PAGE can be accounted for by the presence on human hematopoietic cells of a homologue of murine T200 glycoprotein. In both species, the glycoprotein is a major cell-surface component that is broadly distributed on hematopoietic cells but that has not been detected on other tissues. At present, structural homology between species has only been clearly established for a few other cell-surface glycoproteins of lymphoid cells: immunoglobulin, histocompatibility antigens, Ia, and Thy-1 glycoproteins; and, in the case of Thy-1, there are significant differences in the tissue distribution of the homologous glycoproteins in different species (16–19). T200 glycoprotein, like these other surface glycoproteins, may be present in many species. In the rat, a major cell-surface glycoprotein referred to as leukocyte-common antigen, which is almost certainly a rat homologue of T200 glycoprotein, has been isolated and characterized by Sunderland et al. (20). In both species of rodent, structural polymorphisms of the glycoprotein homologues have been identified; in the mouse the polymorphism is defined by the Ly-5 alloantigenic system (21), in the rat by alloantisera against ART and Ly-1 (22). It is not known whether an analogous polymorphism exists in man. Furthermore, it is not known whether antibodies against the human glycoprotein will generally exhibit selective cytotoxicity for thymocytes and mature T cells, as is the case in rat and mouse (15, 22). Until these points are clarified, the possible clinical uses of monoclonal antibodies against the human homologue cannot be completely assessed.

The results of the peptide-mapping analysis suggests that the structure of T200 glycoprotein is highly conserved between mouse and man. Although a quantitative estimate of homology requires comparison of the primary sequences of the murine and human glycoproteins, the number of overlapping peptides is a good indication of extensive sequence homology. This is particularly true because peptide-mapping analysis tends to underestimate similarities in structure. For example, neither human λ- and κ-immunoglobulin light chains nor the α- and β-subunits of hemoglobulin shares common tryptic peptides even though primary sequence analysis reveals 37% homology between λ- and κ-chains and 44% homology between the subunits of hemoglobin (23–25). It is interesting that, in contrast to Thy-1, Ia and the major histocompatibility antigens, even conventional xenoantisera raised in rabbits, show little or no cross-reactivity between murine T200 glycoprotein and the human and rat homologues (26) (I. S. Trowbridge. Unpublished results.). One possibility compatible with this observation and the peptide mapping data is that a substantial portion of the molecule is highly conserved between species, whereas the remaining fraction, at least part of which is exposed on the cell surface, has undergone extensive modification during phylogeny. The proteolysis experiments suggest the human homologue differs in structure from murine T200 in the region of the molecule external to the plasma membrane, although they give no indication of how extensive these differences may be.

In common with most cell-surface glycoproteins, the functions of T200 glycoprotein and its homologues are not known. The fact that the glycoprotein appears to be restricted to hematopoietic cells in several species strengthens the belief that the molecule serves some role related to the functions of this cell type. We have previously suggested that T200 glycoprotein may be involved in determining the different patterns of migration of murine T and B cells, although there is presently no direct evidence to support this suggestion (26). Kasai et al. (27) have previously reported

that Ly-5 antiserum specifically inhibits murine natural killer activity in vitro in the absence of complement, thus raising the possibility that T200 glycoprotein and its homologues may be involved in cell-mediated cytolysis. Recently, a monoclonal antibody that appears to be specific for the structural form of T200 glycoprotein on activated murine T cells has been obtained (M. Sarmiento. Unpublished results.). This monoclonal antibody, in particular, and those against determinants common to both T and B cells in mouse, rat, and humans that we and others have obtained, should be useful reagents to investigate the biological role of murine T200 glycoprotein and its homologues in other species.

Summary

We report the identification of the human homologue of murine T200 glycoprotein. Peptide-mapping experiments suggest that the structure of the glycoprotein is highly conserved between the two species. Many of the properties of human T200 homologue are similar to those of murine T200 glycoprotein: it is broadly distributed within the hematopoietic system but is not detectable on nonhematopoietic cells; there are also structural differences between the forms of the glycoprotein found on T and B lymphoblastoid cell lines. These results suggest the homologous glycoproteins may play similar functional roles in both species.

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