Brief Definitive Report

IDENTIFICATION OF THE SEQUENCE REQUIRED FOR EXPRESSION OF THE 2H4 EPITOPE ON THE HUMAN LEUKOCYTE COMMON ANTIGENS

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Human T lymphocytes that express the T4 (CD4) cell-surface antigen play a central role in the regulation of immune responses. T4+ lymphocytes are, however, a functionally heterogeneous population of cells that can be subdivided by the mAb anti-2H4, into the T4+, 2H4+ subset that induces suppressor activity of the T8+ suppressor T cells, and the T4+, 2H4- T cell subset with helper function (1). Biochemical analysis has shown that the 2H4 antigens are members of a family of high molecular weight cell surface glycoproteins known as leukocyte common antigens (LCAs), T200 or CD45 (2). While some anti-LCA mAbs react with all forms of LCAs, which are resolved into four or more proteins by SDS-PAGE, other anti-LCA mAbs, including anti-2H4, react only with a subset of these proteins (2–4). In functional studies, anti-2H4, but not anti-LCA mAbs that are reactive with all LCA members, has been shown to block the induction of suppression by T4+, 2H4+ cells (5). These studies suggest that the 2H4 epitope may be directly involved in the induction of suppressor function. It is, therefore, important to establish the molecular basis of the 2H4 epitope expression to understand the function of the suppressor-inducer T cell subset.

Recently, it was demonstrated (6, 7) that the genetic basis of the heterogeneity of the human LCAs is the differential usage of three exons of the single LCA gene. The exons termed A, B, and C, which are differentially used, encode 66, 47, and 48 amino acids, respectively, of the amino-proximal end of the LCA molecules (6). In a previous report (6), cDNA clones corresponding to three LCA structures were isolated, and the existence of at least two more forms of LCA mRNA was shown by Northern hybridization experiments. Since then, we have isolated a cDNA clone that corresponds to one of the predicted structures. In this communication, we study the molecular basis of the 2H4 epitope expression using these cDNA clones encoding four of the LCAs.

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FIGURE 1. Structures of the LCA cDNA subclones used for in vitro RNA synthesis. Thin lines indicate the vector sequence (pSP65 for LCA.6, LCA.260, and LCA.1, and pSP64 for LCA.338), and boxes represent LCA cDNA sequences. The arrows indicate the initiation sites and the direction of RNA synthesis by SP6 RNA polymerase. ATG indicates the initiation site of protein synthesis. Small boxes labeled A, B, or C, are encoded by LCA exons A, B, or C, respectively. Solid boxes indicate the sequences encoding the transmembrane peptide. The four cDNA inserts are incomplete in that they lack the 3’ portions of the protein coding sequences. Therefore, common 3’ ends were created by linearizing the plasmid DNAs by digesting with the restriction enzyme Bgl II.

Materials and Methods

Monoclonal Antibodies. The murine mAbs, anti-2H4, GAP 8.3, and HB7 have been described previously (1, 4, 8).

Plasmid Constructions. The LCA cDNA clones, LCA.6, LCA.260, and LCA.1, were described previously (6). A fourth LCA cDNA clone, LCA.338, was characterized by restriction site mapping and sequence determination (9) (our unpublished results). The LCA.338 cDNA has the same NH2-terminal amino acid coding sequence as LCA.6 except that it lacks the nucleotides that exactly correspond to exon C (6) (Fig. 1). These cDNAs sequences were subcloned into the Eco RI site of the plasmid vector pSP65 or pSP64 (10) such that the 5’ ends of the LCA cDNAs were proximal to the SP6 polymerase transcription start site.

In Vitro Synthesis of LCA mRNA. RNA transcripts were synthesized in a reaction (0.1 ml) containing 10 μg of template SP6.LCA DNA linearized with the restriction enzyme Bgl II, 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 1 mg/ml acetylated-BSA, 1 U/μl RNasin (Promega Biotec, Madison, WI), 0.5 mM each rNTP, 0.5 mM G(5')ppp(5')G (Pharmacia Fine Chemicals, Piscataway, NJ), and 40 U SP6 RNA polymerase (Promega Biotec) for 2 h at 40°C. The full-length RNA products were purified by agarose gel electrophoresis, extracted four times with phenol, precipitated with ethanol, and dissolved in H₂O.

In Vitro Translations. In vitro synthesized LCA mRNAs or Brome Mosaic Virus RNA (Promega Biotec) were translated in a nuclease-treated rabbit reticulocyte lysate according to the procedure recommended by the supplier (Promega Biotec). The translation cocktail (0.1 ml) contained 70 μl of reticulocyte lysate, 20 μM each of 19 amino acids, 1 μM [35S]-methionine (1,100 Ci/mmol), (New England Nuclear, Boston, MA), 1 U/μl RNasin, 2 μg in vitro synthesized RNA, and were incubated for 60 min at 30°C.

Preformed Immune Complex Generation and Immunoprecipitations. Preformed complexes between mAbs and rabbit anti–mouse IgG were prepared by incubating 10 μl of ascites fluid with 12 μg of purified rabbit anti–mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) for 4 h at 4°C. Complexes were recovered by centrifugation (13,000 g for 5 s), and washed four times in cold (4°C) wash buffer (1% (vol/vol) Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mg/ml BSA, 0.1%
Translation cocktails were diluted 20-fold with cold wash buffer containing the following protease inhibitors: 0.2 mg/ml soybean trypsin inhibitor, 2 μg/ml antipain, 2 μg/ml chymostatin, 0.4 μM leupeptin, 0.5 μM pepstatin, 0.3 μM iodoacetamide, 0.2 TIIU/ml aprotinin, and 1 mM PMSF. A 0.2-ml aliquot of the diluted translation cocktail was added to the preformed complexes of anti-2H4 or HB7 antibody and incubated at 4°C for 16 h. Immune complexes were washed four times with cold wash buffer, dissolved in 50 μl of sample buffer (250 mM Tris-HCl, pH 6.8, 2% [wt/vol] SDS, 5% [vol/vol] glycerol, 0.002% [wt/vol] bromophenol blue, and 1% [vol/vol] 2-ME), and boiled for 4 min. Samples were electrophoresed on a 10% SDS-polyacrylamide gel, and autoradiographed as described previously (11).

Radiolabeling and Immunoprecipitation of Cell Surface Molecules. Human T4+ cells were purified from the blood of healthy volunteer donors as previously described (1). 10^9 T4+ cells were labeled with 1 μCi of 125I by peroxidase-catalyzed iodination (12), and solubilized as described previously (11). In the case of preclearing, 0.2 ml of the cell lysate (equivalent of 2 x 10^9 T4+ cells) was precleared five times with either 20 μl of the GAP 8.3 antibody (ascites) bound to 60 μl of a 50% (vol/vol) protein A-Sepharose suspension (Pharmacia Fine Chemicals) or with 50 μl of anti-2H4 antibody crosslinked to Sepharose 4B (equivalent to 25 μg of purified anti-2H4 antibody). For immunoprecipitation, the untreated or the precleared cell lysates were incubated for 3 h with either 5 μl of the GAP 8.3 antibody or 20 μl of anti-2H4 antibody crosslinked to Sepharose 4B. 20 μl of a 50% (vol/vol) protein A-Sepharose suspension were then added to the GAP 8.3 precipitations, and the mixture was incubated for 60 min. The antibody-bound beads were washed two times with 0.5% Triton X-100 in 10 mM Tris-HCl (pH 7.4), 140 mM NaCl, three times with 0.5% deoxycholate in 10 mM Tris-HCl (pH 8.2), 140 mM NaCl, and once with 10 mM Tris-HCl (pH 8.0). Immune complexes were analyzed in reducing conditions by SDS-PAGE on a 6% gel followed by autoradiography (11).

Results and Discussion
The anti-2H4 antibody precipitates proteins of 220 and 200 kD, while the mAbs that react with all LCAs, e.g., GAP 8.3 (4), precipitate 190- and 180-kD proteins as well as 220- and 200-kD proteins from surface-labeled human T4+ cells (4; also see Fig. 3). To study the structural basis for the differential expression of the 2H4 epitope, we generated individual LCA proteins in vitro using cloned human LCA cDNAs (6, 10, 13). The structures of the LCA cDNAs, and the segments of cDNA sequences encoded by the differentially used exons, called exons A, B, or C, are schematically shown in Fig. 1. The cDNA sequences were individually inserted into the plasmid vector pSP65 or pSP64 (10) at a restriction site immediately 3' of the promoter site for the bacteriophage SP6 RNA polymerase. The orientation of the inserts was chosen so that the RNAs synthesized by the SP6 polymerase encode LCA proteins. The four SP6 LCA cDNA plasmids were linearized at the unique Bgl II site, and were used as templates for synthesis of LCA RNA by SP6 RNA polymerase. Since all the LCA cDNA plasmids were linearized at the Bgl II site, the resulting LCA RNAs have exactly the same 3' end, regardless of the 3' end points of cDNA inserts (see Fig. 1). LCA RNAs were purified and used to synthesize LCA proteins in a rabbit reticulocyte lysate system in the presence of [35S]methionine. The in vitro synthesized LCA proteins differ from each other only by the inclusion or exclusion of the amino acids encoded by exons A, B, or C. These proteins are truncated in that they contain only the extracellular portion of the LCA molecules. The translation products of the LCA RNAs, as well as the control Brome Mosaic Virus (BMV) RNA are shown in Fig. 2A.
Immunoprecipitation studies using the in vitro synthesized LCA proteins were carried out to determine which forms of the LCA molecules could be precipitated by anti-2H4 antibody. As seen in Fig. 2B, the anti-2H4 antibody precipitates the LCA.6 and LCA.338 proteins, but does not precipitate the LCA.1 and LCA.260 proteins, or the BMV products. The finding that only LCA proteins that contain the A exon amino acids were precipitated by anti-2H4 argues that the peptide encoded by exon A is necessary for anti-2H4 antibody binding. The control mAb, HB7, with an unrelated specificity but of the same IgG₁ isotype as anti-2H4, did not precipitate any of the in vitro synthesized proteins (data not shown). The Gap 8.3 antibody and several other anti-LCA mAbs did not precipitate any of the LCA proteins synthesized in vitro (data not shown), suggesting that these proteins lack the necessary modification and/or conformation to allow binding by most anti-LCA mAbs. The simplest interpretation of the requirement of exon A expression for the binding of the anti-2H4 antibody to LCA molecules is that the 2H4 epitope is encoded by the A exon. However, we cannot exclude the possibility that the presence of the A exon segment indirectly affects the binding of anti-2H4 to the actual 2H4 epitope residing outside of the A exon segment.

The finding that the expression of LCA exon A is required for the expression of the 2H4 epitope correlates well with our previous Northern hybridization results using an exon A-specific probe (6). While the 2H4⁺ cell lines, Raji and Daudi, strongly express exon A-containing LCA mRNAs, the 2H4⁻ cell line, HSB-2, does not express the A exon (1). The data presented in this paper suggest that the LCA.6 and LCA.338 cDNAs encode the 220- and 200-kD LCA forms, respectively, while the LCA.1 perhaps encodes the 180-kD LCA form. The LCA.260 cDNA, however, can also encode an LCA molecule of about the same size as the LCA.338, i.e., 200 kD. If this is the case, the 200-kD band actually contains at least two forms of LCA molecules, one with the 2H4 epitope (LCA.338) and the other without the 2H4 epitope. To examine this possibility, we did reciprocal preclearing experiments using ¹²⁵I-labeled cell surface molecules (Fig. 3). The preclearing by the GAP 8.3, which reacts to all forms of the LCA molecules, completely removed the anti-2H4-reactive materials. The preclearing by the anti-2H4 antibody completely eliminated the highest molecular weight (220 kD) protein from the GAP 8.3-reactive material, but a substantial amount of the 200-kD protein still remained. Therefore, the 200-kD protein of
the GAP 8.3 immunoprecipitate is indeed heterogeneous, and the second 200-kD LCA molecule lacking the 2H4 epitope does exist. These results, as well as the cDNA cloning, further demonstrate the complexity of the human LCA system.

**Summary**

The anti-2H4 antibody, which subdivides the T4+ population of human T lymphocytes into T4+, 2H4+ suppressor-inducer cells and T4+, 2H4- helper cells, recognizes an epitope on a subset of the human leukocyte common antigens (LCAs). LCAs are a family of cell surface glycoproteins generated from a single gene by the differential usage of three exons near the NH2-terminus. Using cDNA clones corresponding to four of the different forms of LCA molecules, extracellular domains of the LCA molecules were synthesized in vitro. Immunoprecipitation of these molecules with the anti-2H4 antibody demonstrated that exon A is required for the expression of the 2H4 epitope.

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