Human Major Histocompatibility Complex Class II Invariant Chain Is Expressed on the Cell Surface*

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Class II major histocompatibility complex antigens are intracellularly associated with a nonpolymorphic polypeptide referred to as the invariant chain. Before the class II heterodimer appears on the cell surface, the invariant chain dissociates but it has so far been unclear as to whether or not a proportion of the invariant chain also appears on the plasma membrane. We describe a study with three monoclonal antibodies which recognize an extracytoplasmic determinant present on all forms of the invariant chain and use them to demonstrate its presence on the surface of the intact cells. The determinants recognized by two of the antibodies were found to be located within the 60 amino acids at the extreme C-terminal (extracytoplasmic) end of the invariant chain. The invariant chain-specific monoclonal antibody, VIC-Y1, was found to bind a determinant located between amino acids 1 and 73, which correspond to mainly cytoplasmic residues. Using the C-terminal specific antibodies, the number of antibody binding sites on the surface of two B lymphoma lines was estimated to be $10^5$ per cell. The results of this study appear to resolve the highly disputed question of whether or not the invariant chain can appear as a plasma membrane protein. The results are discussed in the context of a possible role for the invariant chain in antigen processing and presentation.

Recognition of an antigenic peptide bound to MHC class II molecules by the T cell receptor of T lymphocytes signals the initiation of an immune response. Class II MHC molecules are polymorphic heterodimers, consisting of noncovalently linked $\alpha$ and $\beta$ chains, both transmembrane proteins. Intra-cellularly, the class II heterodimer is associated with a third, nonpolymorphic polypeptide, the invariant chain (II) (Jones et al., 1978). No specific function has been assigned to the invariant chain as yet, although several observations on the effects of inhibitors (Machamer and Cresswell, 1984; Falo et al., 1987; Rosamond et al., 1987; Sivak et al., 1987) on antigen presentation are consistent with a role of II in this process. More direct evidence for a role of II in antigen presentation was obtained when supertransfection with the invariant chain gene was found to confer enhanced antigen presentation efficiency on class II expressing fibroblasts (Stockinger et al., 1989).

The human invariant chain is a transmembrane protein which associates with the class II molecules in the endoplasmic reticulum (Kvist et al., 1982; Quaranta et al., 1984), but dissociates before the class II molecule appears on the cell surface (Machamer and Cresswell, 1982). Most II remains intracellular, but it is not known how much, if any, occurs on the cell surface, either alone or in association with class II. We have hypothesized that the II molecule contains sorting information for endosomes, and that it may therefore facilitate the transport of class II molecules to a proteolytic compartment (Koch et al., 1989). In this way, the class II heterodimer, either before it reaches the cell surface or after it is recycled, may bind processed antigen.

The invariant chain is inserted into the membrane with its N terminus on the cytoplasmic side. Previously, studies of II cell surface expression have been hampered by the lack of antibodies specific for its C-terminal, extracytoplasmic domain. In particular, VIC-Y1 is shown in this study to recognize an II determinant that is likely to be cytoplasmic. Hence, we have investigated three other monoclonal antibodies and demonstrate here that they are specific for the human invariant chain and recognize its three major forms, II33, II35, and II41, in cell lysates. The antibodies bind to extracytoplasmic determinants of II and thus were used to demonstrate the presence of II on the surface of B cell lines. This finding raises important questions concerning the functional contribution of surface II expression to antigen processing and presentation.

MATERIALS AND METHODS

Antibodies

LN-2 (Epstein et al., 1984) is an IgG1, commercially available from Biotest-Serum-Institut GmbH, West Germany. VIC-Y1 (Quaranta et al., 1984) was kindly provided by Dr. W. Knapp, Vienna, Austria. Monoclonal antibodies BU-43 (IgM) and BU-45 (IgG1) were produced by injecting the B lymphoblastoid cell line, HPB1, four times intraperitoneally, prior to fusion of spleen cells with the AG8.953 myeloma line. Hybridomas were screened for positive reactivity in frozen human tonsil sections. LN-2, BU-43, and BU-45 were included in the coded ("blind") antibody panel of the B cell section of the 4th...
International Conference on Human Leucocyte Differentiation Antigens\(^2\) and were assigned to a new cluster of antibodies. CD74 (Dörken et al., 1989). 2.06 is an anti-class II framework monoclonal antibody (Charroin and McDevitt, 1980), kindly provided by Dr. J. Johnson, Munich, West Germany, and ISCR-3 (Watanabe et al., 1986) is an I\(_2\)G2b specific for an HLA-DR monomorphic determinant, kindly provided by Dr. L. Graf, Seattle. The sera, p14-I and p14-N, have been described previously (Lipp and Dobberstein, 1986a). For the immunoprecipitation, goat anti-mouse Ig fluorescein isothiocyanate conjugate was from the Jackson Laboratories Inc., West Grove, PA, and rabbit anti-mouse Ig was produced by the authors.

Cells

The JOK-1 B cell line, derived from a patient with hairy cell leukemia (Anderson et al., 1982), Raji Burkitt's lymphoma, and U937, a class II negative histiocytic lymphoma line (Sundström and Nilsson, 1976), were cultured in RPMI 1640 medium containing additionally 2 mM glutamine, 10 mM Hepes, 100 \(\mu\)g/ml penicillin, 100 units/ml of streptomycin, nonessential amino acids, and 10% heat inactivated fetal calf serum.

Metabolic Labeling, Immunoprecipitation, and Two dimensional SDS-PAGE

Cells were labeled for 10 min with 200 \(\mu\)Ci of \(^{35}\)S-methionine (Amersham, 800 Ci/mmol) and immunoprecipitated exactly as described previously (Koch and Hämmerling, 1986). Immunoprecipitates were then analyzed by two-dimensional electrophoresis. First dimension nonequilibrated pH gradient gel electrophoresis (NEPHGE) was performed in 20-cm rods as described by O'Farrell (1970), and second dimension SDS-polyacrylamide gel electrophoresis was performed after equilibrating the rods in reducing sample buffer containing 1% SDS and 40 mM dithiothreitol as described.

Production of \(\beta\)-Galactosidase Fusion Proteins

Construction of p\(_{\text{Zi157-216}}\) and p\(_{\text{Zi157-216-m216}}\), an I\(_2\)-icbI fragment of the I\(_{\text{a}}\)41 cDNA encoding the 60 C-terminal amino acids of I\(_{\text{a}}\)33 plus the 64 amino acids of the C-terminal, extracytoplasmic domain. I\(_{\text{a}}\)157-216 contains amino acids 158 to 216 of I\(_{\text{a}}\)33 plus the polylinker region.\(^3\) For the construction of p\(_{\text{Zi157-216}}\), a NcoI fragment of the I\(_{\text{a}}\)41 cDNA encoding the 60 C-terminal amino acids of I\(_{\text{a}}\)33 plus the 64 amino acids derived from alternate splicing of exon 6b of the invariant chain gene (Strubin et al., 1986) in I\(_{\text{a}}\)41 was inserted into a pEX (Stanley and Luzio 1984) derivative which had a NcoI restriction site in the polylinker region.\(^3\)

Expression of I\(_{\text{a}}\)-Galactosidase Hybrid Proteins—Transformed bacteria were grown at 30 °C to an A\(_{5780}\) of 0.1 (Corning calorimeter 1640 medium containing 10% heat-inactivated fetal calf serum, 1% heat-inactivated human serum, and 0.02% sodium azide. After 30

Western Transfer

After separation by SDS-polyacrylamide gel electrophoresis, bacterial proteins were transferred to nitrocellulose membranes overnight at 2 V/cm in 25 mM sodium phosphate, pH 7.0 at 4 °C. The membrane was then soaked in 10 mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl, 3% (w/v) bovine serum albumin for 2 h at room temperature. The blocked membranes were then incubated at 4 °C overnight with 10 \(\mu\)g of monoclonal antibody then washed as described by Burnette (1981). Bound antibody was detected with alkaline phosphatase conjugated goat anti-mouse Ig (10 \(\mu\)g, Jackson Laboratories). 0.8 \(\mu\)g/ml propidium iodide, and analyzed in a Becton Dickinson FACScan flow cytometer.

Cell Surface Invariant Chain

Purified monoclonal antibodies, BU-43 and BU-45, were radiolabeled by the chloramine-T method using 1 mCi of \(^{125}\)Iiodide per 100 \(\mu\)g of monoclonal antibody. Competitive binding inhibition cellular radioimmunoassays were performed in flexible polyvinylchloride microtiter trays. In brief, 50 \(\mu\)l/well target cell suspension, containing 10\(^6\) viable cells diluted in phosphate-buffered saline, 0.2% gelatin, 5% pooled human Ig, were mixed with 50 \(\mu\)l of unlabeled competing antibody at a range of dilutions and allowed to react for 1 h at room temperature. A constant quantity of labeled BU-45, previously determined to be subsaturating from the antibody binding curve for each respective target cell line, was subsequently added (50 \(\mu\)l/well) and allowed to react for a further 1 h at room temperature. After repeated washings by centrifugation and aspiration of the supernatants, the radioactivity in individual wells was measured. Binding of labeled second antibody was calculated as a percentage of the value obtained with 10 \(\mu\)g/well HD37 monoclonal antibody, which reacts with the pan-B cell antigen, CD19, thus serving as an unrelated inhibitor. For the quantitative determination of antigen sites per cell, the same radioimmunoassay was performed. Raji and JOK-1 cells were incubated with increasing amounts (10\(^6\) to 3 \(\times\) 10\(^5\) cpmp) of \(^{125}\)I-labeled BU-43 and BU-45, respectively, for 1 h at room temperature. Monoclonal antibody binding to target cells was analyzed in a Scatchard plot as described previously (Moldenhouer et al., 1987).

RESULTS

Immunoprecipitation of I\(_{\text{a}}\) from Metabolically Labeled Cells—In screening a panel of B cell reactive antibodies, three were found to have a pattern of reactivity on tissue sections similar to that of the invariant chain-specific monoclonal antibody, VIC-Y1. To examine the specificity of the antibodies, U937 cells were lysed after metabolic labeling with \(^{35}\)S methionine, and proteins were precipitated with the monoclonal antibodies, LN-2, BU-43, BU-45, and VIC-Y1. Labeled precipitates were then analyzed in two-dimensional NEPHGE gels. As shown in Fig. 1 (a–c), spots corresponding to the different forms of I\(_{\text{a}}\) (see figure legend) were obtained. Thus, LN-2, BU-43, and BU-45 precipitate all forms of I\(_{\text{a}}\), resulting in a pattern identical to that obtained with VIC-Y1 (Fig. 1d).

Non-specific precipitation in the absence of a primary antibody is shown in Fig. le. The antibodies precipitate I\(_{\text{a}}\) directly rather than through the association of I\(_{\text{a}}\) with class II molecules, since U937 cells are negative for class II, as shown in the immunoprecipitation with the 2.06 anti-class II antibody (Fig. 1f).

Localization of I\(_{\text{a}}\) Determinants Recognized by LN-2, BU-43, BU-45, and VIC-Y1—To determine the parts of the invariant chain molecule recognized by each of the antibodies, cDNA constructs encoding II\(_{\beta}\)-\(\beta\)-galactosidase fusion proteins were expressed in Escherichia coli and analyzed by Western transfer. I\(_{\text{a}}\)-\(_{\text{a}}\)33 contains the 73 N-terminal amino acids of the human invariant chain, which include the cytoplasmic tail, the transmembrane region, and the membrane proximal 16 amino acids of the C-terminal, extracytoplasmic domain. I\(_{\text{a}}\)-\(_{\text{a}}\)-\(_{\text{a}}\) contains the whole C-terminal domain of the 33-kDa form of human I\(_{\text{a}}\) except for the 16-membrane proximal amino acids. I\(_{\text{a}}\)-\(_{\text{a}}\)-\(_{\text{a}}\) contains amino acids 158 to 216 of I\(_{\text{a}}\)33 plus the 64 amino acids encoded by the alternatively spliced exon 6b of the I\(_{\text{a}}\) gene.

As shown in Fig. 2a, LN-2 and BU-43 bind to II fusion proteins after SDS PAGE and Western transfer and recognize only the fusion proteins containing C-terminal, extracytoplasmic segments. Since LN-2 and BU-43 bind to both I\(_{\text{a}}\)-\(_{\text{a}}\)-\(_{\text{a}}\) and I\(_{\text{a}}\)-\(_{\text{a}}\)-\(_{\text{a}}\), the determinants recognized by these antibodies must lie C-terminal to amino acid 156 and must not be contained within the 64 amino acids unique to I\(_{\text{a}}\), since these amino acids are absent in I\(_{\text{a}}\)-\(_{\text{a}}\). Neither LN-2 nor BU-
FIG. 1. Analysis of LN-2, BU-43, BU-45, and VIC-Y1 immunoprecipitates by two-dimensional gel electrophoresis. U937 cells were metabolically labeled with [35S]methionine, lysed, and immunoprecipitated with each antibody as shown. Products were separated in the first dimension by NEPHGE and in the second dimension by SDS-PAGE. Different forms of the invariant chain (Ii33, Ii35, Ii41, p25) are indicated. The different forms originate from the alternate splicing of exon 6b in the Ii gene, giving rise to Ii41, and the initiation of translation at an alternate, upstream, start codon, giving rise to Ii35 (O’Sullivan et al., 1987). The spot marked A is actin. The positions of class II positive cells, are indicated by open circles in f. a–c, putative anti-invariant chain monoclonal antibodies; d, anti-invariant chain monoclonal antibody, VIC-Y1; e, nonspecific immunoprecipitation products in the absence of primary antibody (rabbit anti-mouse Ig only); f, anti-class II (framework) monoclonal antibody, 2.06.

Scatchard Analysis of Cell Surface Antibody Binding Sites—The number of antibody binding sites present on the surface of JOK-1 and Raji cells was estimated by measuring the binding of 125I-labeled BU-45 to intact cells in a radioimmunoassay and by plotting the data for Scatchard analysis. Assuming a molar ratio of 1.0 for antibody to Ii molecules, which most probably represents an underestimate, approximately 1 x 10^5 antigen copies per cell are expressed on both JOK-1 (Fig. 4A) and Raji (Fig. 4B) cells.

Using a similar approach, it was possible to determine the interrelationships of the determinants recognized by the three antibodies. When 125I-labeled BU-43 was incubated with intact JOK-1 and Raji cells after initial binding of unlabeled BU-43, BU-45, or LN-2, LN-2 blocked the binding of BU-43, but BU-45 binding appeared to be sterically independent from that of BU-43 (Fig. 5). Thus, LN-2 and BU-43 bind to the same or overlapping region on Ii, and BU-45 binds to a separate region. This is consistent with the Western transfer data indicating that, unlike LN-2 and BU-43, BU-45 fails to recognize denatured Ii bacterial fusion proteins after transfer to nitrocellulose. BU-45 may therefore recognize a conformational determinant.

DISCUSSION

In this paper we have demonstrated the presence of the human class II-associated invariant chain on the surface of cells. The monoclonal antibodies employed in this study recognize extracytoplasmic determinants on Ii and bind to the surface of cells in flow cytometric analyses. Two of the antibodies, LN-2 and BU-43, recognize overlapping regions within the 60 C-terminal amino acids of Ii33, residues common to all forms of the invariant chain.

A large number of studies have dealt with the question of invariant chain expression on the cell surface, without reaching a common conclusion. In immunoprecipitation experi-
FIG. 2. Analysis of antibody binding to partial Ii fusion proteins after Western transfer. a, three β-galactosidase fusion proteins containing segments of the invariant chain were expressed in E. coli, separated by 7.5% SDS-PAGE, and transferred to nitrocellulose filters. The filters were then incubated with LN-2, BU-43, BU-45, or VIC-Y1, as shown. Binding of antibody was detected with alkaline phosphatase-conjugated goat anti-mouse antibodies. A, Ii, containing the 60 amino acids at the extreme C-terminal end plus the alternatively spliced segment (64 amino acids) encoded by exon 6b; B, Ii, containing the cytoplasmic tail, membrane-spanning region, and 16 amino acids on the C-terminal (extracytoplasmic) side of the membrane-spanning region; C, Ii, containing all of the C-terminal domain except for the 16 membrane proximal amino acids. E. coli proteins before electrophoretic transfer, stained with Coomassie Blue, are shown in the right hand column. Bands corresponding to Ii fusion proteins, as determined with anti-β-galactosidase antibodies (data not shown), are indicated with an arrow. b, summary of antibody binding to partial Ii fusion proteins.

ments using antibodies directed against class II molecules with ¹²⁵I-labeled surface proteins (Shackleford and Strominger, 1980; Charron and McDevitt, 1980) or with partially purified membrane preparations (Sung and Jones, 1981; Moosic et al., 1982), no Ii was found in the plasma membrane. When analogous studies were performed using antibodies specific for Ii itself, conclusions about the intracellular distribution of Ii also differed according to the antibody or method used. The VIC-Y1 antibody does not stain intact B cells (Quaranta et al., 1984; this study), an observation that may be consistent with our finding that VIC-Y1 binds to a largely cytoplasmic segment of Ii. The Ii-specific polyclonal antibody, R184, produced negative results in both flow cytometric analyses and in immunoprecipitations of surface-labeled cells (Accolla et al., 1985), whereas another monoclonal antibody, mcAb 21:9, appeared to give positive results in indirect immunofluorescence microscopy (Claesson-Welsh et al., 1986). The binding of the monoclonal antibody, In-1, to lymphocyte tumor cell lines (Koch et al., 1982) most probably represented binding to internal structures, accessible on dead, nonintact cells, since In-1 was later shown to recognize an exclusively cytoplasmic determinant of murine Ii (Lipp and Dobberstein, 1986a).

One possible explanation for the varying conclusions of previous studies on Ii surface expression is the previous absence of antibodies directed against C-terminal portions of Ii. Another cause could be that Ii is expressed on the surface of only a limited number of cell types. In addition to the B cell lines, Raji and JOK-1, other cell types were tested for expression of Ii on their surfaces by flow cytometry. The B cell
Burkitt lymphoma line, Daudi, exhibited a level of Ii surface expression similar to that of Raji. Antigenic determinants reactive with LN-2, BU-43, and BU-45 were also observed on other lymphoblastoid cell lines, resting and activated peripheral blood B cells, and in pre-B and progenitor B cell neoplasias (Dorken et al., 1989). The class II-positive T cell line, HUT-78, was negative for surface Ii despite the high cytoplasmic content of Ii indicated in immunoprecipitates of whole cell lysates.

The results of the present study show conclusively that Ii appears on the surface of B cells. What, then, is the function of the invariant chain? Several observations concerning the co-expression of Ii and Ia, including tissue distribution (Momburg et al., 1986), and co-induction by γ-interferon (Koch et al., 1984; Momburg et al., 1986), plus the association of Ii with class II molecules during its assembly (Kvist et al., 1982), strongly suggest that Ii expression is linked to class II function. However, the proposal that Ii is required for the transport of class II molecules to the plasma membrane (Sung and Jones, 1981; Kvist et al., 1982) is not supported, since cells transfected with class II in the absence of Ii transport class II heterodimers to the surface (Sekaly et al., 1986; Miller and Germain, 1986).

Does the invariant chain have a role in antigen presentation? We recently suggested that the invariant chain contains a sorting signal for endosomal compartments (Koch et al., 1989). The complex of class II molecules and Ii could then be transported to an endosomal compartment where limited proteolysis occurs. Here, a proteolytic cleavage could separate Ii from the class II heterodimer (Blum and Crosswell, 1988), and, in the same compartment, class II could associate with processed antigen. Having lost its sorting signal, class II would then appear on the cell surface. The hypothesis that Ii contains an endosomal sorting signal is supported by the observation that Ii localizes in endosome-like structures, even in the absence of class II.

Does the cell surface form of Ii have a specific function? One possibility is to facilitate the recycling of cell surface class II molecules to processing compartments inside the cell. Observations that cycloheximide inhibits antigen processing in adherent peritoneal exudate cells, but not in B lymphoma hybridomas (Harding and Unanue, 1989), support the notion that cell surface invariant chain can facilitate the recycling of cell surface class II molecules, since Ii surface expression appears to be characteristic of B-lineage cells. Without surface Ii, newly synthesized class II may be the only vehicle for the presentation of processed peptide on the cell surface.

Another recent observation, that the cell surface adhesion molecule, ICAM-1, restores the antigen presentation function of L-cells (Altmann et al., 1989), raises the possibility that hitherto unidentified cellular accessory molecules come into play during antigen presentation under physiological conditions. Such a role could be postulated for cell surface invariant chain.

In conclusion, the antibodies described in this paper provide a means for studying the functional significance of cell surface Ii. In particular, correlation between antigen presentation function in subsets of B cells and their level of Ii surface expression can now be investigated. The well defined binding sites of the antibodies also makes them suitable for a characterization of the intracellular trafficking of Ii.

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