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Immunology

Subunit structure of cell surface proteins: Disulfide bonding in antigen receptors, Ly-2/3 antigens, and transferrin receptors of murine T and B lymphocytes

(cell surface immunoglobulin/T-cell receptor/receptor aggregation/transmembrane signaling)

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ABSTRACT **The surface proteins of lymphocytes from spleen and thymus and several cultured lymphoid tumor lines were radioiodinated *in situ*, solubilized with Triton X-100, and examined for the presence of disulfide-bonded subunits by two-dimensional (intact, reduced) NaDodSO$_4$/polyacrylamide gel electrophoresis. [Hynes, R. O. & Destree, A. (1977) *Proc. Natl. Acad. Sci. USA* 74, 2855–2859]. Few lymphocyte surface proteins were found to consist of disulfide-bonded subunits, and the most prominent of these could be identified. In normal B lymphocytes and B-lymphoma cells, IgD or IgM (or both) were the major disulfide-bonded proteins, and these were easily detectable, even without immunoprecipitation. In contrast, analysis of thymocytes and T-lymphoma cells did not reveal any protein resembling immunoglobulin in its chain structure. The major labeled thymocyte membrane protein consisting of disulfide-bonded subunits was identified as the Ly-2/3 antigen. It appeared to contain disulfide-bonded homodimers of M_r 35,000 (α_2) noncovalently associated with a second pair of homodimers of M_r 30,000 (β_2). Peptide mapping showed these polypeptides to be homologous. A third disulfide-bonded homodimer, which was heterogeneous in apparent M_r, appeared to be part of the Ly-2/3 complex. All cultured T- and B-lymphoma lines examined were found to possess a major surface protein that appeared to be a disulfide-bonded homodimer of a polypeptide of M_r 95,000. This protein was identified as the receptor for transferrin. It is suggested that the presence of two or more subunits in cell surface receptors renders their ligand functionally bivalent, making ligand-induced receptor aggregation possible.**

Very little is known about the nature of the receptor for antigen on thymus-derived (T) lymphocytes (1, 2). Virtually all efforts to understand its structure have examined the question of serological crossreactivity between the T-cell receptor and immunoglobulin (1–4). This approach has yielded little firm biochemical information, although many reports have suggested a disulfide-bonded chain structure reminiscent of immunoglobulin (2, 3).

The presence of interchain disulfide bonds in proteins can be shown by a form of two-dimensional polyacrylamide gel electrophoresis in which the proteins are intact in the first dimension but reduced in the second (5). Proteins that are not disulfide bonded will lie on a diagonal, and the subunits of disulfide-bonded proteins will lie on vertical lines below the diagonal. We have used this form of gel electrophoresis to ask the following questions. (*i*) How common is interchain disulfide bonding in lymphocyte membrane proteins? (*ii*) Can two-dimensional gel electrophoresis reveal surface immunoglobulin on B lymphocytes without the use of immunoprecipitation? (*iii*) Do T lymphocytes possess detectable amounts of surface proteins that resemble immunoglobulins in their chain composition and disulfide bonding?

Our results indicate that few strongly labeled lymphocyte membrane proteins consist of disulfide-bonded subunits and that the majority of these can be identified. Immunoglobulin is easily detectable in the membranes of B lymphocytes, but a protein resembling immunoglobulin in its disulfide bonding is not detectable in T lymphocytes. The use of intact/reduced electrophoresis also provides new information about the structure of the Ly-2/3 antigens and cell surface receptors for transferrin.

MATERIALS AND METHODS

Normal Lymphocytes. Spleen cells and thymocytes were prepared from 5- to 6-week old female BALB/c or CBA mice as described (6).

Tumor Cell Lines. The B-lymphoma line WEHI-231.1 (7), the plasma cell tumor line MPC-11.45.6.2.4 (8), and the T-lymphoma lines EL-4.1, WEHI-7.1, WEHI-22.1, and STRij-4-2.2 (ST-4) (7) were maintained in Dulbecco's modified Eagle's medium/10% fetal calf serum.

Antisera. Rabbit anti-mouse polyclonal Fab fragment was as described (6). Monoclonal antibodies to Ly-2 antigen (clone 49.11.1) were kindly provided by M. Hogarth, University of Melbourne.

Cell Surface Radioiodination. Cells were prepared and labeled with 500 μCi of Na^{125}I (1 Ci = 3.7 × 10^{10} becquerels) as described (6).

Solubilization of Membrane Proteins. Labeled cells (5×10^7) were suspended in 200 μl of ice-cold 100 mM Tris·HCl, pH 7.4/1 mM *N*-ethylmaleimide/1 mM phenylmethylsulfonyl fluoride/1 mM EDTA/0.5% Triton X-100. After 15 min at 4°C, nuclei were removed by centrifugation at 2,000 × *g* for 5 min. Two hundred microliters of 5% NaDodSO$_4$ in H$_2$O was added, and the sample was placed in a boiling water bath for 5 min.

Immunoprecipitation. Triton extracts were held at 4°C for 1 hr with appropriate amounts of antibody, and immune complexes were isolated by standard procedures (6).

Precipitation of Transferrin-Binding Protein. Cell lysates were held at 4°C for 30 min with 50 μl of agarose beads to which had been coupled iron-loaded human transferrin (Sigma). Beads were then washed once in 5 ml of phosphate-buffered saline, pH 6, and bound proteins were eluted by boiling in nonreducing sample buffer.

Two-Dimensional Polyacrylamide Gel Electrophoresis. The first dimension consisted of a Laemmli tube gel (7.5% acrylamide) (9). Tubes were siliconized to facilitate gel removal. Sample volume was 50–100 μl, and gels were run at 1 mA per

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Abbreviation: ST-4, STRij-4-2.2 (cells).

gel, constant current. When the bromophenol blue marker reached the bottom, the gel was removed and equilibrated for 2 hr at 25°C in 5 ml of Laemmli sample buffer/300 mM dithiothreitol. The second dimension consisted of a Laemmli slab gel (10% acrylamide). Reduced gels contained 5 mM sodium thioglycolate in the upper gel reservoir buffer. Other details were essentially as described (6).

Peptide Mapping by Limited Proteolysis. Radioactive proteins were analyzed by a slight modification of the method of Cleveland *et al.* (10). Areas of dried gels containing the α and β subunits of the Ly-2/3 antigens were identified by autoradiography, cut out with scissors, placed on the stacking gel (3% acrylamide) of a 15% slab gel, and overlaid with sample buffer/20 mM dithiothreitol containing papain at 300 μg/ml. Current was applied until the dye approached the separating gel, turned off for 2 hr at 37°C, and then resumed until the dye reached the end of the gel.

RESULTS

Disulfide Bonding in Spleen Cell Membrane Proteins. Spleen cells from CBA mice were radioiodinated, solubilized, and analyzed by two-dimensional gel electrophoresis. Control experiments in which both dimensions were intact (Fig. 1*A*) or reduced (Fig. 1*B*) showed little deviation from a smooth curve relating the mobility in the first dimension to that in the second, indicating that there was no significant reoxidation of reduced disulfide bonds or disulfide interchange during electrophoresis.

When the proteins were intact in the first dimension but reduced in the second (Fig. 1*C*), a small number were seen to have dissociated into their subunits. Immunoprecipitation analysis (Fig. 1*D*) identified monomeric IgM (μ_2L_2), monomeric IgD (δ_2L_2), and IgD half molecules (δ_1L_1), all of which were clearly visible without immunoprecipitation, and traces of unreduced IgM and IgD. As previously reported (6), the δ chains showed some heterogeneity in molecular weight, while the μ and light chains ran as sharp bands. Immunoglobulin was removed quantitatively from the extract by immunoprecipitation, and the remaining material was analyzed. No additional bands were found to have been obscured by the immunoglobulin heavy or light chains (data not shown).

A protein of M_r 200,000 intact/95,000 reduced was sometimes detectable (e.g., Fig. 1*C*). It was much more prominent on dividing cells (see below).

Disulfide Bonding in Surface Proteins of Thymocytes. Thymocytes from CBA mice were labeled and analyzed in the same way as spleen cells (Fig. 2). Few major proteins were found to consist of disulfide-bonded subunits, and there was no detectable protein resembling immunoglobulin in its chain composition (Fig. 2*A*).

Two labeled proteins fell below the main line, one of M_r 65,000 intact/35,000 reduced and another of M_r 60,000 intact/30,000 reduced. This pair of proteins resembled the Ly-2/3 antigens, which are present on ≈85% of thymocytes (11). Their identity as Ly-2/3 was confirmed by specific immunoprecipitation with monoclonal anti-Ly-2 antibody. Examination of the supernate after precipitation showed depletion of these proteins (Fig. 2*B*). Peptide maps of the M_r 35,000 and 30,000 spots were remarkably similar (Fig. 2*A Inset*). A third, diffuse, spot (Fig. 2) also appeared to be part of the Ly-2/3 complex.

As with spleen cells, thymocytes sometimes gave detectable amounts of the protein of M_r 200,000 intact/95,000 reduced (Fig. 2; see below).

Disulfide Bonding in Surface Proteins of Cultured T- and B-Cell Lines. The surface proteins of several cultured cell lines were also examined by two-dimensional gel electrophoresis (Fig. 3). The B-lymphoma line WEHI-231 was found to possess very large amounts of surface IgM in the monomeric form (μ_2L_2) and also larger polymeric forms (Fig. 3*A*). The intensity of the IgM spots was so great that it was necessary to use very short exposures (10 hr) to prevent excessive blackening of the film. In contrast, the T-lymphoma line ST-4 (Fig. 3*B*) and several

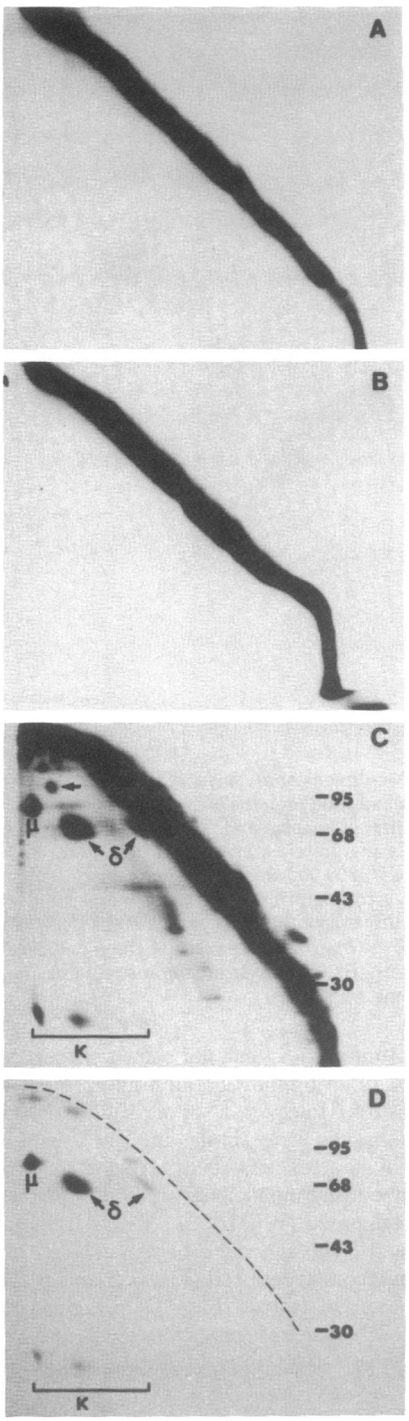

FIG. 1. Two-dimensional polyacrylamide gel electrophoresis of ^{125}I-labeled membrane proteins of murine spleen cells. Electrophoresis proceeds from left to right and then from top to bottom. Numbers, $M_r \times 10^{-3}$. (*A*) Both dimensions intact. (*B*) Both dimensions reduced. (*C*) First dimension intact, second dimension reduced; horizontal arrow, position of the M_r 200,000/95,000 protein. (*D*) Immunoprecipitated surface immunoglobulins—first dimension intact, second dimension reduced; dotted line, position of proteins that do not consist of disulfide-bonded subunits.

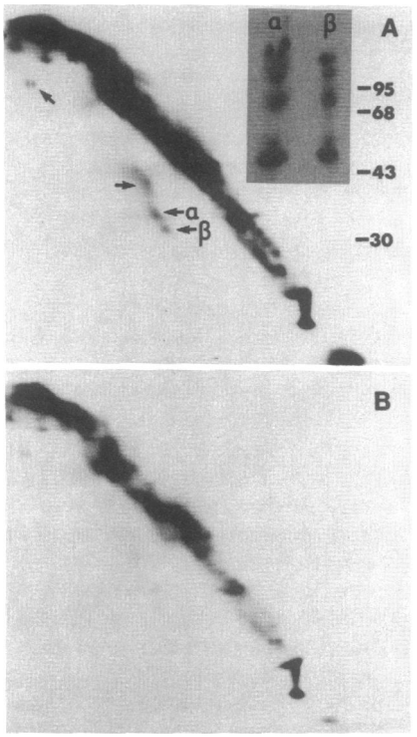

FIG. 2. Two-dimensional polyacrylamide gel electrophoresis of ^{125}I-labeled membrane proteins of murine thymocytes. Proteins were intact in the first dimension and reduced in the second. (*A*) Total thymocyte membrane proteins; arrow pointing upward and to the left, position of the M_r 200,000/95,000 protein; α and β, subunits of the Ly-2/3 antigens; arrow pointing to the right, a consistently observed but unidentified diffuse spot that may be part of the Ly-2/3 complex; numbers, $M_r \times 10^{-3}$. (*Inset*) Peptide maps of the α and β subunits of the Ly-2/3 antigens. (*B*) Thymocyte membrane proteins after depletion of Ly-2/3 antigens.

other T-cell tumor lines (data not shown) did not yield detectable amounts of an immunoglobulin-like protein even after 3 days of exposure. However, a set of disulfide-bonded proteins of ST-4 membranes were similar but not identical to Ly-2/3 proteins of thymocytes. ST-4 cells are Ly-2 positive by immunofluorescence using monoclonal antibodies (data not shown).

All lines examined (WEHI-231, MPC-11, EL-4, WEHI-7, WEHI-22, and ST-4) showed a heavily labeled protein of M_r 200,000 intact/95,000 reduced (Fig. 3 *A* and *B*). This protein bound to transferrin-agarose (Fig. 3*D*) but not to unconjugated agarose (Fig. 3*C*) or agarose conjugated with unrelated proteins (data not shown). It is thus identified as the receptor for transferrin.

DISCUSSION

IgM and IgD Are the Major Disulfide-Bonded Surface Proteins of Spleen Cells. Our results show that surprisingly few labeled proteins from spleen cell membranes consist of disulfide-bonded subunits. Monomeric IgM and IgD and half-molecules of IgD were the major proteins in this category. Once the positions of these proteins after two-dimensional gel electrophoresis had been established, detection was possible without immunoprecipitation. Apart from the Ly-2/3 proteins, there was no evidence for a molecule that resembled immunoglobulin in chain composition and disulfide bonding on thymocytes or T-cell lymphomas.

The failure to detect a protein on the surface of T cells that resembles immunoglobulin in its disulfide bonding could be due to inadequate labeling, solubilization or sensitivity, or proteolytic degradation. Another possibility is that the T cells examined here do not possess antigen receptors; this question could be addressed by analysis of functional T-cell lines. Even if the T-cell receptor is immunoglobulin-like, it may not consist of disulfide-bonded subunits. The finding of unusual disulfide bonding (and sometimes none) between the heavy chains of surface IgD provides a precedent for this possibility (6, 12).

Ly-2/3 Antigens Are the Major ^{125}I-Labeled Disulfide-Bonded Surface Proteins of Thymocytes. On thymocytes, a major pair of labeled polypeptides possessing interchain disulfide bonds was identified as the Ly-2/3 antigens. A similar (but not identical) set of proteins was observed in ST-4 cells.

The Ly-2/3 antigens have been reported to consist of polypeptides of M_r 35,000 and 30,000 (13, 14). We suggest that these polypeptides be named α chains and β chains, respectively. Peptide mapping (Fig. 2*A Inset*) shows that the α and β chains must possess considerable sequence homology. Whether they are separate gene products or different glycosylated forms or proteolytic cleavage products remains to be determined. A third, rather diffuse, spot was also observed in thymocytes (Fig. 2) and was immunoprecipitated with the Ly-2/3 complex. It was apparently replaced by a homogeneous spot in ST-4 cells (Fig. 3). This may represent a previously unrecognized component of the Ly-2/3 complex.

The quaternary structure of the Ly-2/3 antigens is still the subject of debate (13, 14). Our data show that the α and β chains of Ly-2/3 from thymocytes are separable before reduction and suggest that each exists in the form of a disulfide-bonded homodimer (Fig. 2). A heterogeneous third disulfide-bonded homodimer, which we tentatively name γ, also appears to be part of the Ly-2/3 complex.

The Ly-2/3 proteins are of interest for several reasons. Among immunologically functional T cells, they are confined to suppressor T cells and killer T cells and their precursors (15). Antibodies against the Ly-2/3 proteins inhibit certain T-cell functions (16). The *Ly-2/3* loci are closely linked to the κ immunoglobulin locus (17), but they would appear unlikely to code for the antigen receptor of T cells because, although their products are heterogeneous in charge, this has been ascribed to variability in sialic acid content (13). The nature of the diffuse spot seen in the present work, however, has not been determined. The possibility still remains that a component of the Ly-2/3 complex is the receptor, and the symmetry between immunoglobulin and Ly-2/3 antigen expression on B and T cells may be more than fortuitous.

Receptor for Transferrin Is the Major Disulfide-Bonded Surface Protein of Cultured T-Cell Lines. All cultured cell lines tested (T, B, and plasma cell) were found to possess a surface protein of M_r 200,000 intact/95,000 reduced. This protein was the major disulfide-bonded protein on all four T-cell lines examined, but since it was not restricted to T cells, it was an unlikely candidate for the antigen receptor of T cells. Lymphoid cell lines have recently been found to possess substantial numbers ($\approx 10^5$ per cell) of specific binding sites for the iron-transport protein transferrin (18, 19). The protein that has transferrin-receptor activity from rabbit reticulocytes (20) and human placenta (21) has an M_r of 200,000 intact/95,000 reduced. Thus, it seemed likely that the protein with these characteristics from the cell lines of the present study was the transferrin receptor, and this was confirmed by demonstrating its specific binding to iron-loaded transferrin–agarose (Fig. 3).

There have been several other recent reports of cell surface proteins that may be identical with the transferrin receptor. One of these is a protein present on human erythroblasts and erythroleukemia cells but not on erythrocytes, granulocytes, or

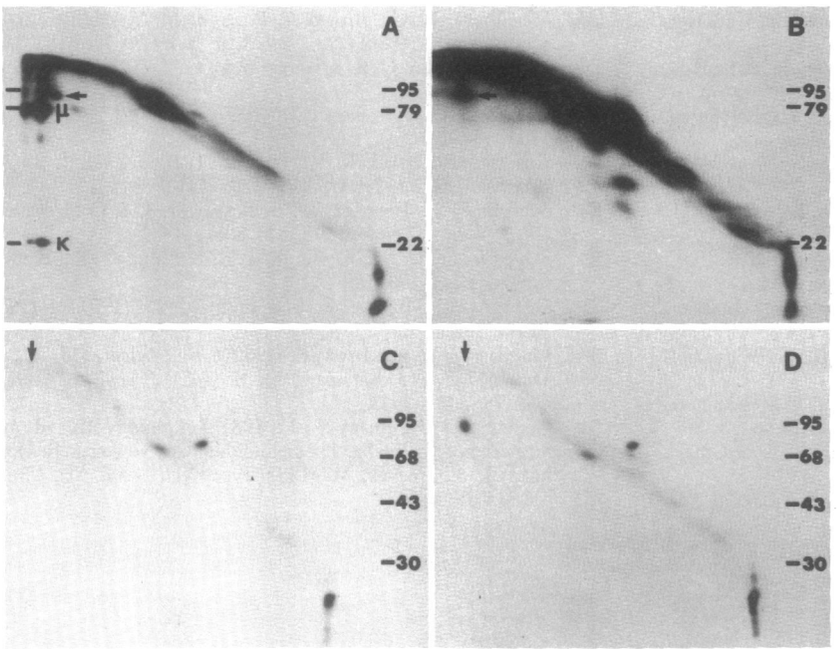

FIG. 3. Two-dimensional polyacrylamide gel electrophoresis of ^{125}I-labeled membrane proteins of lymphoid tumor cells. Proteins were intact in the first dimension and reduced in the second. Numbers, $M_r \times 10^{-3}$. (*A*) B-cell lymphoma WEHI-231. Positions of immunoglobulin μ and κ chains were verified by immunoprecipitation (not shown); horizontal arrow, position of the M_r 200,000/95,000 protein. (*B*) T-cell lymphoma ST-4. Horizontal arrow, position of the M_r 200,000/95,000 protein. (*C*) Membrane proteins from ST-4 cells bound to and eluted from unconjugated agarose. Vertical arrow, position of myosin (M_r 200,000). (*D*) As for *C*, but using transferrin-conjugated agarose.

macrophages (22). Another is a protein identified by its reaction with certain antisera and with monoclonal antibodies OKT9 and 5E9 and demonstrable on various types of dividing but not on nondividing cells (23). An additional candidate is a glycoprotein of reduced M_r ≈100,000 shown to be associated with malignancy (24, 25).

Comparative peptide mapping after limited proteolysis with staphylococcal V8 protease suggests identity of transferrin receptors with the weakly labeled disulfide-bonded protein of similar electrophoretic properties seen in some experiments with spleen cells and thymocytes (unpublished data).

Biological Significance of Subunit Structure of Membrane Receptors. Membrane receptors frequently consist of two or more subunits. Virtually all of the well-studied lymphocyte surface proteins suspected to be involved in specialized immunological function (immunoglobulin, H-2 antigens, Ia antigens, and Ly-2/3 antigens) consist of two or more subunits. The insulin receptor (26) and the transferrin receptor are also made up of disulfide-bonded subunits.

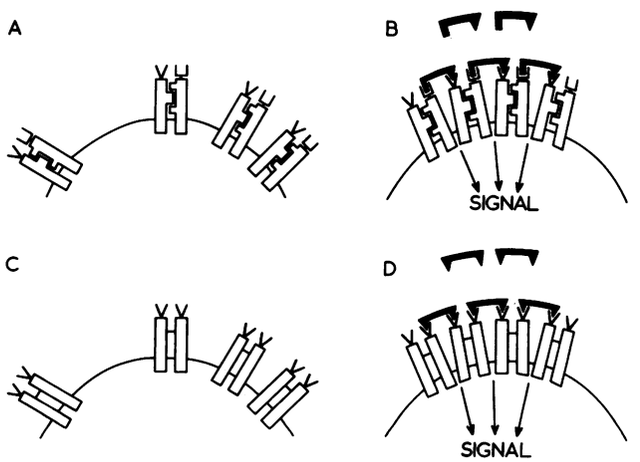

FIG. 4. Possible role of subunits of membrane receptors in ligand-induced crosslinking. (*A* and *B*) The receptor is made up of two nonidentical subunits, each of which binds to one portion of the ligand. (*C* and *D*) The receptor is made up of two identical subunits, and the ligand possesses two identical receptor-binding sites.

Subunit structure may be due to the separation of recognition properties on one subunit from effector function on the other. We have recently postulated such a role for H-2 and β_2-microglobulin (27). Alternatively, multiple subunit structure may reflect a need for crosslinking of receptors for transmembrane signaling (28–31). In other words, the ligand may have two distinct receptor-binding sites, one for each subunit of receptor (Fig. 4). The ligand binding sites would be arranged to make it sterically impossible for one ligand to bind simultaneously to both sites on one receptor. In this way, crosslinking of receptors could be achieved by apparently univalent ligands. Transferrin consists of two highly homologous domains (32), and its receptor consists of two apparently identical subunits. The interaction of transferrin with its cell surface receptors may therefore represent a paradigm of the concept of divalent ligand–receptor interactions.

Note Added in Proof. Trowbridge and Omary (33) have shown that the human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. Monoclonal antibody OKT9 has been shown to react with the human transferrin receptor (34, 35).

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