[26] Assembly of Histocompatibility Antigens

By BERNHARD DOBBERSTEIN and SUNE KVIST

Histocompatibility antigens are oligomeric proteins expressed on the plasma membrane. Two classes of these antigens can be distinguished: transplantation, or class I, antigens, and immune associated, or class II, antigens.[1] Class I antigens are composed of a glycosylated, membrane-spanning heavy chain that is noncovalently associated with β_2-microglobulin, a peripheral membrane protein.[1,2] They are called H-2, K, D, and L antigens in mouse and HLA-A, B, C antigens in man. Class II antigens consist of three glycosylated membrane-spanning proteins that are noncovalently associated.[3] In mouse they are called Ia antigens; in man, HLA-DR antigens.[1] Their polymorphic subunits are the α (35 kilodaltons) and β (29 kilodaltons) chains, and the invariant one is the I or γ (33 kilodaltons) chain.[4]

Both types of histocompatibility antigens are synthesized on membrane-bound ribosomes and cotranslationally inserted into the membrane of the endoplasmic reticulum.[5-10] Here they are proteolytically processed and glycosylated, and their subunits, which are synthesized on separate mRNAs, are assembled into an oligomeric complex. During intracellular transport their carbohydrate portions are modified.[5,9,10] In the case of the HLA-DR antigens, DRγ chains detach from the oligomeric complex before it reaches the cell surface.[10]

Biosynthesis and cell surface expression of these antigens can be studied *in vitro* and *in vivo*.[5-10] *In vitro* the events occurring in the endoplasmic reticulum can be reconstructed, i.e., cotranslational insertion, and even oligomeric assembly.[10] Selected methods to study biosynthesis and membrane insertion of histocompatibility antigens are described here.

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ISBN 0-12-181996-5

Choice of Cells to Study Biosynthesis of Histocompatibility Antigens

H-2 antigens are present in largely varying amounts in different tissues or cells.[11] Thus the proper choice of the cell type used for the study is important. Thymoma cells like the SL2 cells (H-2^d haplotype) express relatively large amounts of H-2 antigens on their cell surface. They are grown in ascites form in DBA/2 mice (about 0.5 to 1×10^9 cells per mouse). The human lymphoblastoid cell line Raji is a rich source of HLA-DR antigens. Both cell types contain low amounts of ribonuclease and can be efficiently biosynthetically labeled.[5]

Isolation of mRNA Coding for Histocompatibility Antigens

To study biosynthesis of histocompatibility antigens *in vitro,* a fraction enriched in mRNA coding for H-2 or HLA antigens must be isolated. This can be done by purifying messenger RNA (mRNA) from rough microsomes. The same procedure can be used for both cell types.

SL2 cells are grown in ascites form in DBA/2 mice (Bomholtgord, Denmark) and harvested from the peritoneal cavity 8–10 days after inoculation with 2×10^6 cells. The cells are washed 3 times in ice cold 50 m*M* Tris-HCl, pH 7.5, 100 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* dithiothreitol (DTT), and 100 μg of cycloheximide per milliliter, swollen in 20 m*M* Tris-HCl, pH 7.5, for 10 min, and then broken using a Dounce homogenizer. Isotonic conditions are established by adding an equal volume of 20 m*M* Tris-HCl, pH 7.5, 300 m*M* KCl, 5 m*M* $MgCl_2$, 100 μg of cycloheximide per milliliter. Nuclei and mitochondria are removed by centrifugation at 8000 *g* for 10 min. From the resulting supernatant, crude rough microsomes are pelleted by centrifugation for 1 hr at 20,000 rpm in a Sorvall SS34 rotor. RNA is extracted from the pelleted microsomes by the phenol–chloroform–isoamyl alcohol method, and poly$(A)^+$ RNA is purified on oligo(dT)-cellulose.[12] mRNA coding for H-2 antigens is thus enriched about 10-fold.[5] Further purification can be conveniently achieved by centrifugation in an 8 to 20% aqueous sucrose gradient.[5] Such a separation is usually sufficient to obtain a further 5- to 10-fold enrichment.

Immunocharacterization of H-2 and HLA-DR Antigens

The major problem in the characterization of histocompatibility antigens synthesized *in vitro* or *in vivo* is that they represent only a very

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minor proportion of cellular proteins. Furthermore, antigenic determinants present on the mature protein might not be found on the one synthesized in a cell-free system. Like many membrane proteins, histocompatibility antigens are modified during intracellular transport. The signal sequence is removed, and they become glycosylated and assembled into an oligomeric complex. Some of these modifications are essential for antigenic determinants to become expressed and must be considered in the immunocharacterization.

Immunoprecipitation Protocol

After translation of SL2 mRNA in a cell-free system (25 μl) supplemented with dog pancreas microsomes, the following operations are performed at 4°. Ribosomes are dissociated by adding 75 μl of 10 m*M* EDTA, 150 m*M* KCl, 20 m*M* Tris-HCl, pH 7.5, and the membranes are pelleted by centrifugation at 30 psi for 10 min in a Beckman Airfuge. Antigens present in the pelleted membranes are then solubilized in 50 μl of Nonidet P-40 (NP-40) buffer [1% NP-40, 10 m*M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 2 m*M* EDTA, 10 μg of phenylmethylsulfonyl fluoride (PMSF) per milliliter] and transferred to a 1.5-ml Eppendorf tube. One microliter of a nonimmune serum and 50 μl of a 1 : 1 slurry of protein A–Sepharose (Pharmacia, Uppsala) in NP-40 buffer are added. Incubation is performed for 30 min under slow rotation of the tube on a Denley Mixer (England). Beads and nonsolubilized material are then removed by centrifugation for 10 min in an Eppendorf centrifuge. The supernatant is incubated for 30 min with the appropriate immune serum (5 μl in the case of an alloantiserum and 1 μl when a heteroserum is used), and then 50 μl of a 1 : 1 slurry of protein A–Sepharose NP-40 buffer are added. This is allowed to react for 2 hr as above. Beads are then washed 3 times with a 1-ml portion of NP-40 buffer, once with NP-40 buffer containing 0.5 *M* NaCl, and once with 20 m*M* Tris-HCl, pH 7.5. After all supernatant fluid has been carefully removed, 25 μl of sample buffer are added, and proteins are characterized by PAGE[13] and fluorography.[14]

H-2 antigens are synthesized in a cell-free system (derived from reticulocytes) as higher molecular weight precursors that are not recognized by most of the alloantisera used to identify H-2 antigens from different loci or haplotypes. When, however, H-2 antigens are inserted into microsomal membranes from dog pancreas, glycosylated and their signal sequence cleaved, the H-2D^d molecule can be specifically detected by an anti-H-2D^d alloantiserum.[5]

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Characterization of Oligomeric HLA-DR Antigens

In the case of HLA-DR antigens the DRγ chain is not recognized by an HLA-DR antiserum even after insertion of the chain into microsomal membranes. For its detection, assembly with DRα and DRβ chains is required.[10]

To achieve the oligomeric assembly of HLA-DR chains *in vitro,* it is necessary for the three different subunit chains to be inserted into the same microsomal vesicle. An alternative is fusing the microsomal vesicles together after translation is completed so that the subunit chains can laterally diffuse in the membrane and assemble. We tested membrane concentrations between 0.06 and 0.25 A_{280} unit of microsomal membranes in a 25-μl assay system. Assembly was observed only at the lowest microsome concentration. This concentration might be different for each antigen and mRNA studied, as it depends largely on the amount of specific mRNA coding for each subunit. Furthermore, in the case of HLA-DR antigens, a very favorable situation exists, as the DRγ chain is synthesized in large excess over DRα and DRβ chains.

Often it is desirable to obtain the separated subunits of an oligomeric protein complex in order to test the antigen specificity of an antiserum. In the *in vitro* system containing large amounts of membranes, exclusively native monomeric subunit proteins are synthesized. Thus it can be conveniently used in the characterization of antibodies raised against subunit chains. The advantage in this procedure is that oligomeric protein complexes need not be disassembled, and hence the subunits are not denatured. This could be particularly useful in the characterization of monoclonal antibodies to determine the subunit chain with which they react.

Assembly of HLA-DR Antigens *in Vivo*

H-2 and HLA-DR antigens are assembled from subunits synthesized in largely different amounts. β_2-Microglobulin is synthesized in excess over the H-2 heavy chain, and the DRγ chain in excess over the DRα and DRβ chains. In each case the unassembled subunits, β_2-microglobulin or the DRγ chain, remain in the endoplasmic reticulum and the oligomeric protein complex travels to the cell surface.

To follow oligomeric assembly of HLA-DR antigens during intracellular transport, two types of pulse–chase experiments can be performed: one in which unlabeled protein is allowed to be synthesized during the chase period, and one in which it is stopped, but after 100 min allowed to proceed again. The assembly process is followed by an antiserum that precipitates the DRγ chain only after it has assembled with DRα and DRβ chains.[10] Such an analysis will allow one to determine requirements for oligomeric assembly and intracellular transport of DR chains.

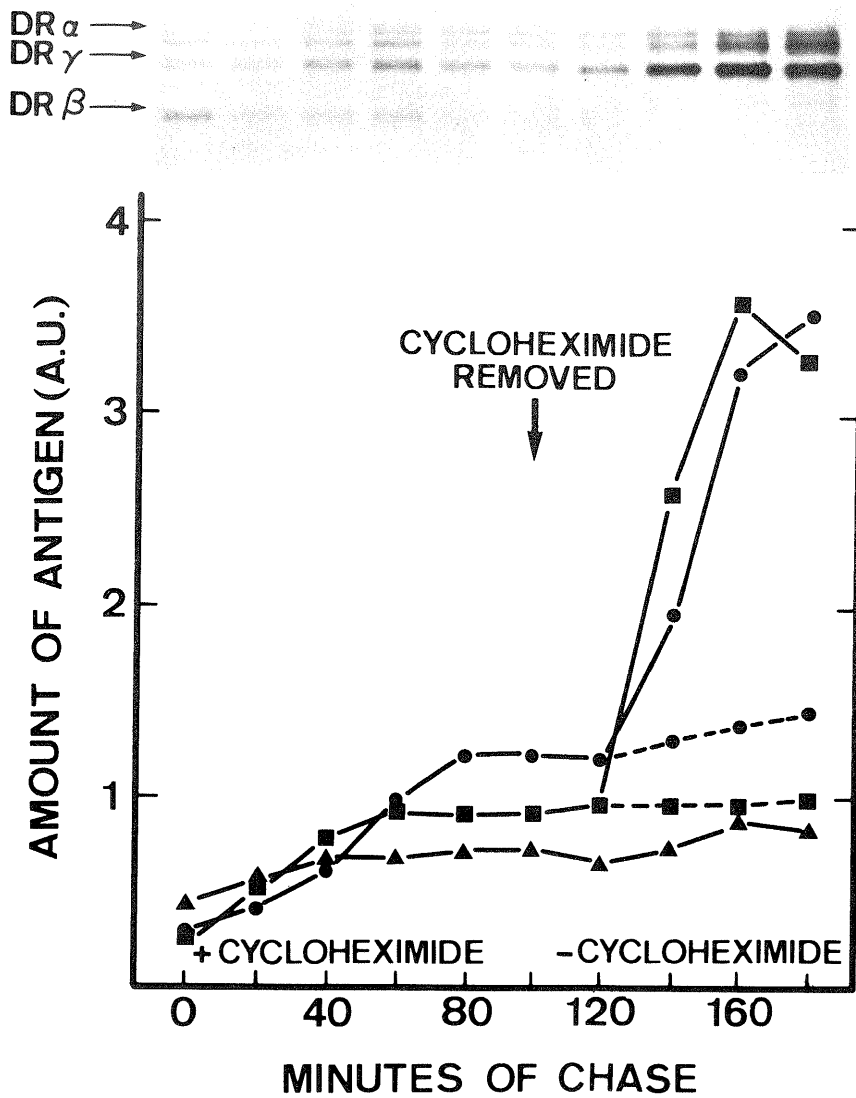

FIG. 1. Assembly of DRγ chains with DRα and DRβ chains. Raji cells are pulse-labeled for 10 min and then chased for 100 min in the presence of cycloheximide. Intracellular transport of membrane proteins proceeds under these conditions. After 100 min, cycloheximide is removed and unlabeled proteins are allowed to be synthesized again. At the time points indicated, [^{35}S]methionine-labeled antigens are immunoprecipitated with the anti HLA-DR antiserum, which precipitates the DRγ chains only after it has complexed with DRα and DRβ chains. Antigens are characterized by SDS–polyacrylamide gel electrophoresis and quantitated by densitometry. ▲——▲, DRβ chains; ■——■, DRγ chains; ●——●, DRα and processed DRγ chains. The dashed lines show the amounts of antigen precipitated when cycloheximide was not removed after 100 min of chase.

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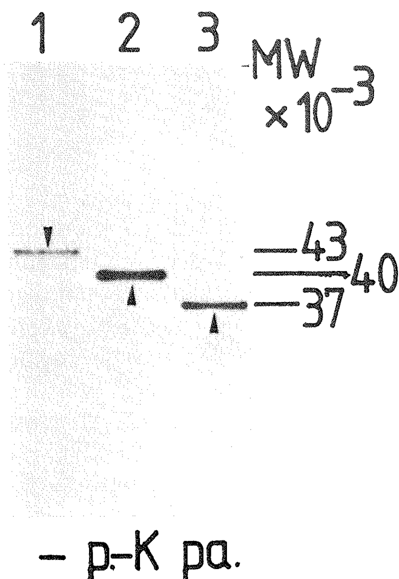

FIG. 2. Determination of the size of the membrane-spanning segment of an H-2D antigen. H-2D antigens, pulse labeled *in vivo*, are characterized by SDS–polyacrylamide gel electrophoresis and autoradiography after no proteolytic digestion (lane 1), after treatment of microsomes with proteinase K (lane 2), and after papain treatment of solubilized antigens (lane 3).

Procedure. The human lymphoblastoid cell line Raji is cultured in RPMI-1640 medium, 10% fetal calf serum. Cells are washed 3 times in methionine-free RPMI-1640 medium, and 1×10^7 cells/ml are incubated for 15 min at 37° to lower the endogenous methionine pool. [^{35}S]Methionine (250 μCi; 1000 Ci/mmol) is then added, and the cells are incubated for 10 min. Then 10 ml of RPMI-1640 complete culture medium and 10 m*M* methionine are added, and aliquots of 1 ml (1×10^6 cells) are removed after various times of incubation. To stop synthesis of unlabeled proteins after the 10-min pulse, cycloheximide is added to a final concentration of 100 μg/ml. To remove cycloheximide after the 90 min of chase, cells are washed twice at 0° with 10 ml of RPMI-1640 medium.

Cells removed at various time points are pelleted and solubilized in 100 μl of 50 m*M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 1% Triton X-100, and 40 μg of PMSF per milliliter. Particulate material is removed by centrifugation at 100,000 *g* for 15 min at 4° in an Airfuge. Antigens are characterized from the supernatant by immunoprecipitation and PAGE (Fig. 1). It can be concluded from this experiment that DRγ chains are synthesized in excess over DRα and DRβ chains.

Determination of the Size of the Membrane-Spanning Segment

Proteins that span the membrane often can be proteolytically cleaved close to the membrane. A papain cleavage site exists in the case of H-2,

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K, D, L[2], and HLA-DR[3] antigens that removes the N-terminal portion in a soluble form. This cleavage can be performed either on the intact cell or on the detergent-solubilized proteins. In each case the same size fragment is liberated. The size of the cytoplasmically disposed portion of a membrane-spanning protein can be estimated by cleaving it with proteinase K. Comparing the sizes of the proteins generated after each of these cleavages will allow one to estimate the approximate length of the membrane-spanning portion. The size thus estimated was in good agreement with the stretch of uncharged residues found by sequence analysis (Fig. 2).

Procedure. SL2 cells (5×10^6) are labeled in 1 ml of RPMI medium for 10 min with 200 μCi of [^{35}S]methionine, washed, and broken as described above. Nuclei are removed by centrifugation at 4000 *g* for 10 min, and the resulting 1-ml supernatant is incubated at 4° for 90 min with 200 μg of proteinase K per milliliter. The reaction is terminated by the addition of PMSF (40 μg/ml) and serum albumin (10 μg/ml). Membranes are pelleted by centrifugation at 100,000 *g* for 20 min. After solubilization of the pelleted microsomes, antigens are immunoprecipitated as described above.

For papain cleavage of H-2 antigens, microsomes from SL2 cells (5×10^6) labeled for 10 min with [^{35}S]methionine are solubilized in 200 μl of 1% NP-40, 100 m*M* NaCl, and 0.25 mg of cysteine per milliliter and treated for 30 min at 37° with 0.5 mg of papain per milliliter. Proteolysis is terminated by the addition of neutralized iodoacetic acid to a final concentration of 0.5 mg/ml. Antigens are immunoprecipitated and characterized by PAGE and fluorography (Fig. 2).

[27] Biosynthesis and Intracellular Transport of Acetylcholine Receptors

By DOUGLAS M. FAMBROUGH

The acetylcholine receptors of vertebrate skeletal muscles and the electric organs of certain fish are multisubunit, integral membrane proteins that become cation-selective ion channels in response to the binding of acetylcholine. In normal adult muscles and electric organs, the acetylcholine receptors are located almost exclusively in the postsynaptic membrane at points of functional contact with nerves. The regulation of acetylcholine receptor number and distribution has been of considerable interest to neurobiologists for several decades in relation to denervation

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ISBN 0-12-181996-5