Membrane Insertion and Oligomeric Assembly of HLA-DR Histocompatibility Antigens

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Summary

HLA-DR histocompatibility antigens are assembled in the endoplasmic reticulum. This assembly has been studied in vitro and in vivo. Three polypeptides are involved in forming the oligomeric structure of HLA-DR antigens. DRα chains (molecular weight 35,000), DRβ chains (molecular weight 29,000) and DRγ chains (molecular weight 33,000). They are cotranslationally inserted into the membrane of the endoplasmic reticulum, and all span the membrane. The size of the cytoplasmic portion of DRα and DRβ is about 500–1000 daltons, whereas that of the DRγ chain is about 3000 daltons. Oligomeric assembly of DRα, DRβ and DRγ chains occurs shortly after their synthesis in the endoplasmic reticulum. DRγ chains are synthesized in excess of DRα and DRβ chains, and hence in the endoplasmic reticulum they are found either in a complex with DRα and DRβ or in a free form. Free DRγ chains remain in the endoplasmic reticulum, whereas DRγ chains present in the oligomeric complex with DRα and DRβ undergo intracellular transport. Their molecular weight increases during transport, probably because of the addition of complex sugars in the Golgi complex. This is followed by the detachment of DRγ chains from the oligomeric complex and the appearance of DRα and DRβ chains on the cell surface. Whether any DRγ chains appear on the cell surface is uncertain.

Introduction

Plasma membrane proteins such as the histocompatibility antigens are first inserted into the membrane of the endoplasmic reticulum and then transported via the Golgi complex to the cell surface (Palade, 1975; Vitiella and Uhr, 1975; Dobberstein et al., 1979; Krangel et al., 1979; Owen et al., 1980, 1981). Many of these proteins are oligomeric, and their biosynthesis and assembly can be envisaged to occur in two different modes. They can be synthesized from one mRNA as a large precursor protein that is subsequently cleaved, as is true for sucrose isomaltase (Hauri et al., 1979). Alternatively, the subunits could be synthesized from separate mRNAs and subsequently assembled into an oligomeric complex. In the latter case, the question arises of how this assembly process is regulated and coordinated. Since assembly into oligomeric complexes is essential for the surface expression of histocompatibility antigens (Ploegh et al., 1979; Algranati et al., 1980; Lee et al., 1980; Owen et al., 1980), they are an excellent model system to study this question.

We describe membrane insertion, assembly and intracellular transport of human HLA-DR histocompatibility antigens. They are oligomeric cell-surface glycoproteins composed of three different polypeptides translated from separate mRNAs (Lee et al., 1980). They are essential for immune responsiveness, activation of lymphocytes in the mixed lymphocyte reaction and stimulation of T cells (Katz and Benacerraf, 1976). They are homologous at the structural and functional level to mouse la antigen (Gipart et al., 1977; Klageskog et al., 1977; Klein, 1979; Silber et al., 1979). HLA-DR antigens on the cell surface comprise a heavy chain DRα (molecular weight 35,000) noncovalently associated with a light chain DRβ (molecular weight 28,000) (Klageskog et al., 1979; Snary et al., 1977; Springer et al., 1977a and 1977b). It is not clear whether one or both of these chains are polymorphic (Klageskog et al., 1978a; Silver and Ferrone, 1979; Charron and McDevitt, 1980; Korman et al., 1980; Shackelford and Strominger, 1980). Both chains span the plasma membrane close to the carboxy terminus, leaving the large amino-terminal end on the extracellular side (Walsh and Crampton, 1977; Kaufman and Strominger, 1979). A third polypeptide, invariant and basic in its character, is found associated with HLA-DR (Shackelford and Strominger, 1980; Charron and McDevitt, 1980) as well as with la antigens (Jones et al., 1978; Moosig et al., 1980). This polypeptide (DRγ) has a molecular weight of about 31,000–33,000 daltons and is thought to occur only in an intracellular form (Shackelford and Strominger, 1980). Its relationship to the molecules found on the cell surface is unclear. We report on the oligomeric assembly of HLA-DR antigens and their cell-surface expression. Special attention has been paid to the invariant polypeptide DRγ and its assembly with the DRα and DRβ chains.

Results

Characterization of in Vitro and in Vivo Synthesized and Membrane-inserted HLA-DR Antigens

Since all of our data depend on the specificity of the antisera used, it was necessary to determine precisely which polypeptides the antisera are directed against. For oligomeric complexes this is particularly difficult because an antibody to one of the proteins in the complex may precipitate the others. To test the spec-
ificity, we took a novel approach based on the fact that in vitro there is a vast excess of microsomal vesicles over membrane proteins synthesized (Blobel and Dobberstein, 1975). Hence an average of only one protein is present per vesicle. It was thus possible to test the specificity of each antibody on the monomeric membrane proteins.

Three antisera were used. A rabbit anti-HLA-DR antiserum was raised against the native HLA-DR complex. It recognizes exclusively HLA-DR antigens when tested against B-cell-surface markers (Klareskog et al., 1978b). The two other sera, the rabbit anti-HLA-DRα and the rabbit anti-HLA-DRβ antisera were raised against the separated HLA-DR antigen subunits.

These sera were used to precipitate antigens synthesized in the cell-free system containing mRNA from Raji cells and microsomal vesicles capable of inserting nascent membrane proteins (Katz et al., 1977; Shields and Blobel, 1978; Dobberstein et al., 1979). The anti-DR antiserum precipitated polypeptides of two size classes, HLA-DRα chains with an apparent molecular weight of 35,000 daltons and HLA-DRβ chains of 29,000 daltons (Figure 1, lane 1). DRα and DRβ chains were both doublets. This was not unexpected, since at least two populations of DR molecules exist on Raji cells (Lampson and Levy, 1980). These polypeptides were membrane-associated and glycosylated, for they sedimented with the membrane fraction during centrifugation and could be bound to lentil lectin (data not shown). The anti-DRβ antiserum precipitated the HLA-DRβ subunit, and the anti-DRα antiserum precipitated the HLA-DRα subunit (Figure 1, lanes 2 and 3). These antisera react only with those subunits to which the antibodies were raised. A rabbit nonimmune serum did not precipitate any antigens (data not shown).

To study the oligomeric assembly of HLA-DR antigens in the rough endoplasmic reticulum, we labeled Raji cells for 10 min with 35S-methionine. The cells were lysed in physiological buffer containing 1% of the detergent Triton X-100. Newly synthesized HLA-DR antigens were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. In contrast to the results obtained with cell-free system, the anti-DR antiserum precipitated three distinct proteins, the DRα chain (molecular weight 35,000), the DRβ chain (molecular weight 29,000) and a third polypeptide with a molecular weight of 33,000 (Figure 1, lane 4). This polypeptide is assumed to be homologous to the invariant basic protein (DRγ chain) first described by Jones et al. (1978). The DRγ chain was not precipitated from the cell-free system even though it was synthesized (see below). Hence, in vivo, it must have associated with the DRα and the DRβ chains in the rough endoplasmic reticulum and coprecipitated with the anti-DR α serum was used.

The anti-DRβ antiserum precipitates only the HLA-DRβ chains (Figure 1, lane 5). In contrast, the anti-

![Figure 1. Membrane Insertion of HLA-DR Antigens in Vitro and in Vivo](image)

Messanger RNA from Raji cells was translated in a reticulocyte cell-free system in the presence of microsomal membranes derived from dog pancreas. After translation, membranes were pelleted, and antigens were immunoprecipitated and characterized by SDS-polyacrylamide gel electrophoresis and autoradiography. For in vivo characterization, Raji cells were labeled with 35S-methionine for 10 min, lysed, immunoprecipitated and characterized as described above. Antigens were precipitated with an anti-HLA-DR antiserum (lanes 1 and 4), an anti-HLA-DRβ antiserum (lanes 2 and 5) and an anti-HLA-DRα antiserum (lanes 3 and 6).
the DRα subunit could be precipitated by the subunit antisera immediately after the pulse. Twenty minutes later not more than 1% of the DRβ and 3% of the DRα subunits were detected (Figure 2). This suggests that oligomeric assembly of HLA-DR antigens occurs soon after their synthesis, probably in the rough endoplasmic reticulum. The larger percentage precipitated with the DRα antisera might reflect the notion that this serum recognizes a DRα–DRγ complex, whereas the DRβ antisera recognizes exclusively monomeric DRβ chains.

**Oligomeric Assembly of HLA-DR Antigens in Vitro**

Since the DRγ chain was precipitated with the anti-DR serum only when associated with the DRβ or DRα chains, its presence in the immunoprecipitate would indicate that oligomeric assembly had occurred. In the cell-free system described above the DRγ chain was not detected, suggesting that oligomeric assembly had not occurred. This was probably due to the large number of microsomal vesicles. For oligomeric assembly to occur, it would be necessary for different subunit proteins to be inserted into the same vesicle. By decreasing the number of membrane vesicles in the cell-free system, we could increase the probability that several different DR subunit molecules would be inserted into the same vesicle.

Messenger RNA from Raji cells was translated in the reticulocyte lysate system in the presence of increasing amounts of microosomal vesicles, and newly synthesized HLA-DR antigen subunits were characterized by SDS-polyacrylamide gel electrophoresis. Figure 3 shows that the presence of the DRγ chains in the anti-DR precipitate was a function of the number of microsomal vesicles. The DRγ chain was precipitated only when the number of vesicles was low (Figure 3, lane 1). Thus the cell-free system can also be used to study the oligomeric assembly of membrane proteins.

**All HLA-DR Antigen Subunits Span the Membrane**

Walsh and Crumpton (1977) and Kaufmann and Strominger (1979) showed that HLA-DR antigens span the membrane. Their analysis, however, did not discriminate between DRα and DRγ chains and did not allow an estimate of the size of that portion of the DR chains on the cytoplasmic side of the membrane. We determined the size of this portion by proteolytic digestion of microsomal vesicles in which HLA-DR antigens had been inserted. Proteinase should digest all those parts of newly synthesized membrane proteins that are located on the cytoplasmic side of the membrane. Those parts of the molecule that have traversed the membrane should be shielded by the membrane. Raji cells were labeled for 10 min, the microsomes were isolated and HLA-DR antigens were immunoprecipitated either directly or after treatment...
with proteinase K. All HLA-DR antigens immunoprecipitated after this treatment had a lower molecular weight (Figure 4, consult lanes 1 and 2), showing that all HLA-DR subunits span the membrane. On the cytoplasmic side, 500–1000 daltons of the DRα and DRβ antigens and about 3000 daltons of the DRγ chain were present. When the membranes were disrupted by detergent before treatment with proteinase K, all HLA-DR subunit chains were digested (not shown).

Processing and Assembly of HLA-DR Antigens during Intracellular Transport
Plasma membrane proteins synthesized on ribosomes bound to the endoplasmic reticulum have to be transported to the cell surface. For many membrane glycoproteins this transport is accompanied by processing of the asparagine-linked carbohydrate moiety from a mannos-rich to a complex-type oligosaccharide. For H-2K and H-2D antigens (Dobberstein et al., 1979) and HLA-A, -B and -C antigens (Krangel et al., 1979) and for vesicular stomatitis virus glycoprotein (Katz et al., 1977), such a change in carbohydrate structure results in an increased molecular weight, which can be seen by SDS-polyacrylamide gel electrophoresis. All DR subunit chains become glycosylated in the endoplasmic reticulum by asparagine-linked carbohydrate units (Charron and McDevitt, 1980). We studied their modification during intracellular transport and the concomitant oligomeric assembly of the DR chains. Raji cells were pulse-labeled with 35S-methionine for 10 min, then chased with an excess of cold methionine. At the times indicated in Figure 5, a sample of cells was withdrawn and solubilized, and the antigens were precipitated with the anti-DR antiserum. The DRβ chains, with an apparent molecular weight of about 29,000, were modified during the chase period to slower moving forms of about 30,000 daltons. A quantitation of the DRβ chains showed that the amount, after an initial increase, stayed constant over the 5 hr chase (Figure 5). A much more complex picture was evident for the DRα and the DRγ chains. The DRγ protein increased dramatically during the chase period, displaying its strongest appearance at about 60 min after the pulse and then declining rapidly (Figure 5). Proteins in the region of the DRα antigens showed a comparable increase, but the optimum appearance was reached at about 90 min after the pulse, or about 30 min later than for the DRγ protein. This time lag suggests that during intracellular transport the DRγ chain is processed to a larger form which is indistinguishable from the DRα protein. After 90 min of chase, the proteins in the region of 35,000 daltons, DRα and DRγ chains, were reduced, probably as a result of dissociation of DRγ chains from DRα and DRβ. This reduction was in clear contrast to the constant amount of DRβ chains during the same period.

In contrast to the three forms found intracellularly only two major bands, both doublets, were detected on the cell surface; one with a molecular weight of about 30,000 corresponding to the DRβ antigens, and
one at about 36,000. The 36,000 dalton proteins correspond not only to the DRα chain but also to processed DRγ protein (data not shown).

In order to follow only the DRα chains during intracellular transport, it was necessary to remove, selectively, the DRγ chains from the immunoprecipitates. This can be achieved by washing the immunoprecipitates in buffer containing low concentrations of SDS (Shackelford and Strominger, 1980). The amount of DRα and DRβ chains then precipitated with the DR serum during intracellular transport is shown in Figure 6. In contrast to the precipitation without SDS, the amount of protein found during the chase in the region of the DRα chains was constant. This indicates that processed DRγ and DRα chains have a similar molecular weight of about 35,000 to 36,000 and are indistinguishable by SDS-polyacrylamide gel electrophoresis.

No antibodies in the anti-DR serum were directed against the DRγ protein itself, thus the increase in this protein during the chase was probably a result of its association with DRα and DRβ chains. Since the chase was performed with excess cold methionine and with continued protein synthesis, excess labeled DRγ protein might, during the period of chase, have combined with unlabeled DRα or DRβ chains. This possibility was tested by performing the chase under conditions where further protein synthesis was blocked but intracellular transport was not affected. Cycloheximide is effective in blocking protein synthesis rapidly and is known to have no effect on intracellular transport of membrane proteins (Jamieson and Palade, 1968). Furthermore, its effect is reversible.

Raji cells were pulse-labeled with 35S-methionine and immediately after were treated with cycloheximide and cold methionine and chased for 100 min at 37°C. To analyze DR antigens, samples were removed at the times indicated in Figure 7. After 90 min, cells were cooled on ice and divided into two aliquots. From one aliquot cycloheximide was removed by washing the cells at 0°C in medium containing excess methionine. Both cell samples were separately chased for another 80 min, aliquots were withdrawn at the times indicated in Figure 7 and the amounts of DR antigens were determined.

During the first 100 min of chase the relative amounts of the three subunit proteins stayed almost equal. No drastic increase of any DR subunit was seen, in contrast to results obtained under conditions
of continuing protein synthesis (see Figure 5). The same result was seen in the second 80 min of chase when the sample was kept in cycloheximide. In contrast, in the samples where cycloheximide was removed after the first 100 min of chase, a dramatic increase in the 33,000 molecular weight protein (DRγ) region was seen, followed by a similar increase in the 35,000 molecular weight protein region. The most likely explanation for this observation is that labeled DRγ chains have associated with unlabeled DRα or DRβ antigens synthesized after the removal of the cycloheximide.

Discussion

Oligomeric assembly of HLA-DR antigens was studied by use of antisera that recognize the assembled HLA-DR complex, DRα subunit chains or DRβ subunit chains. We established the specificities of these antisera using monomeric antigens synthesized in vitro. The monomeric nature of these antigens was ensured by the amount of microsomal vesicles in the cell-free system. This method for analyzing antisera against oligomeric proteins has the advantage that protein complexes need not be dissociated by denaturing agents, which often lead to destruction of antigenic sites.

HLA-DR antigens, found intracellularly after short pulse-labeling, consisted of three size classes of polypeptide chains: 35,000 dalton (DRα), 29,000 dalton (DRβ) and 33,000 dalton (DRγ). The DRα and DRβ chains could be detected by heterologous antisera in their monomeric forms, whereas the DRγ chain was only detected by its association with either DRα or DRβ chains. The DRγ chain has all the properties of the invariant basic polypeptide described by Charron and McDevitt, 1980, and Jones et al., 1978. Since this chain only coprecipitated with DRα and DRβ chains, it has been suggested that it is associated nonspecifically with them, and would thus be similar to actin, which is found as a contaminant in many immunoprecipitates (Shackelford and Strominger, 1980). The results presented here suggest that the DRγ chains do not assemble nonspecifically with DRα and DRβ chains during lysis with detergent (Moosic et al., 1980). This is evident from two types of pulse-chase experiments. Increasing amounts of DRγ chains are precipitated only when, during the period of chase, unlabeled proteins are allowed to be synthesized. As the same amount of labeled proteins is synthesized in both types of chase experiments, artifactual association of DRγ chain with DRα and DRβ during cell lysis should result in an equal amount of DRγ being precipitated regardless of the chase condition. Since this was not the case, assembly of DRγ with DRα and DRβ chains occurs in the native membrane and is not a solubilization artifact.

Oligomeric assembly of the DR subunit chains occurs in the rough endoplasmic reticulum. After a pulse-label of 5 min, more than 70% of the subunit antigens are assembled into oligomeric complexes. This assembly is accompanied by certain changes, since specific antisera, which recognize the free subunits, do not recognize the assembled ones. The DRγ chain in the rough endoplasmic reticulum combines in a noncovalent manner with the DRα chain and the DRβ chain. One intermediate form in the assembly pathway was found to be a DRα-γ complex (see Figure 1, lane 6). Additional evidence that the association of the DRγ chain with DRα or DRβ occurs within the rough endoplasmic reticulum came from cell-free systems where the amount of microsomal vesicles was limiting.

Many membrane glycoproteins are modified during intracellular transport to forms containing complex sugars. This modification occurs in the Golgi complex (Green et al., 1981). Our data suggest that all the HLA-DR subunit proteins reach the stage where the complex sugars are added. The molecular weight of the DRβ chains increases during intracellular transport from 29,000 to 30,000 daltons. The apparent molecular weight of the DRγ chains increases from 33,000 to about 35,000 daltons. All DR subunit chains span the membrane of the endoplasmic reticulum. Only a few amino acid residues of the DRα and DRβ chains extend on the cytoplasmic side. This is in agreement with data obtained by Owen et al. (1981). In contrast, a considerably larger portion of the DRγ chains, approximately 20–30 amino acid residues, extends on the cytoplasmic side.

For proteins composed of subunits synthesized from separate mRNAs, it is important to understand how assembly and intracellular transport of such oligomers takes place and is regulated. In the case of certain immunoglobulins, the light chain is produced in excess over the heavy chain, and only oligomeric, light chain–heavy chain complexes can be efficiently secreted by the cell (Shapiro et al., 1966; Bergman and Kuehl, 1979; Tartakoff and Vassalli, 1979). A similar transport requirement exists for the HLA-A, -B, and -C histocompatibility antigens (Algranati et al., 1980; Owen et al., 1980; Ploegh et al., 1981; Sege et al., 1981). In the case of HLA-DR antigens, the DRγ chain is synthesized in excess over the DRα and the DRβ chains, as shown by experiments where pulse-labeled cells were chased in the presence of cycloheximide to prevent further protein synthesis. Intracellular transport of existing antigens to the cell surface continued under these conditions, and labeled DRα and DRβ chains were transported out of the endoplasmic reticulum. If DRγ subunits were produced in excess, there should still be residual, radioactively labeled DRγ chains which could associate with nascent DRα and DRβ chains once the cycloheximide block had been removed. This was indeed the case. It would then appear that once a DRα chain or
a DRβ chain is inserted into the endoplasmic reticulum membrane, it combines with the available DRγ chain, and this complex travels to the cell surface. The excess DRγ chains might actually help nascent DRα and DRβ chains to become assembled. Since the amount of DRγ chain is greatly reduced during the chase, it may remain at an intracellular location or be degraded. The ultimate fate of the DRγ chain remains to be established. In the mouse la system, it has been shown that the Eβ chain is necessary for the surface expression of the Eγ chain (Jones, 1980). Whether a similar requirement exists for the DRα and DRβ chains remains to be investigated.

The determinants involved in the regulation of intracellular transport of membrane proteins are poorly understood. For certain membrane glycoproteins a clear requirement for their carbohydrate portion exists (Gibson et al., 1980). For others, for example, HLA-A, -B and -C antigens, the presence of the carbohydrate is not necessary for intracellular transport (Owen et al., 1980). Similarly, oligomeric assembly might be essential for certain proteins to be transported to the cell surface and might actually regulate the cell-surface expression of some of them. It is conceivable that proteins such as β2-microglobulin or one of the HLA-DR subunit chains confer a property on the oligomeric protein complex that allows determinants to become effective that are necessary for intracellular transport. However, perhaps only one of the HLA-DR subunits might be endowed with such determinants and could thereby function as a carrier protein. In this respect it is interesting to note that the DRβ chain is found associated not only with DRα and DRβ chains but also with a 41,000 dalton protein (S. Kvist, unpublished results). Although the HLA-DR antigens are a complex mixture of proteins, they are an excellent model system to study oligomeric assembly of membrane proteins and the regulation of their cell-surface expression. Our data suggest that oligomeric assembly is one of the means by which the cell regulates and provides conditions for the cell-surface expression of plasma membrane proteins.

Experimental Procedures

Cell Cultures and Labeling of Cells

The human lymphoblastoid cell line Raji was cultured in RPMI-1640 medium containing 10% fetal calf serum, 50 μg kanamycin/ml and 1.0 μg amphotericin B (Fungizone)/ml.

Before labeling, cells were washed three times in methionine-free RPMI-1640 medium. In a typical labeling procedure cells (1 × 10⁶ in 500 μl) were incubated at 37°C for 10 min in methionine-free RPMI-1640 medium containing 250 μCi of ³⁵S-methionine (500 Ci/mole). When pulse-chase experiments were performed, 10 ml of RPMI-1640 complete culture medium was added immediately after the pulse. Aliquots of 1 ml (1 × 10⁶ cells) were removed at the times indicated in the figure. The radioactivity was incorporated into proteins after the pulse was assayed by determining the total ³⁵S-methionine incorporation for each sample removed during the chase period. When cycloheximide was used to block protein synthesis, it was added at a concentration of 100 μg/ml to pulse-labeled cells. Cycloheximide was removed by washing cells twice at 0°C.

Labeled cells were solubilized in 50 mM Tris–HCl buffer (pH 7.4), containing 0.15 M NaCl, 2 mM EDTA and 1% Triton X-100. The protease inhibitor PMSF (Sigma) was added to a final concentration of 40 μg/ml. After 30 min on ice, particulate material was removed by centrifugation at 100,000 × g for 20 min at 4°C. The supernatant was used for immunoprecipitation of labeled antigens.

Antisera and Immunoprecipitation

The rabbit anti-DR antiserum was raised against native, highly purified HLA-DR antigens. The antigens were purified from spleens taken from patients with chronic lymphocytic leukemia. The purification schedule has been described (Klareskog et al., 1974). The anti-DRs and anti-DRβ antisera was obtained by injecting DR subunits separated by SDS–polyacrylamide gel electrophoresis into rabbits. All three antisera were produced by repeated injections of the lymph node and footpad. The rabbit antisera against HLA-A, -B and -C antigens and against β2-microglobulin have been characterized elsewhere (Rask et al., 1976). Labeled solubilized antigens were first treated with 2 μl of normal rabbit serum and 50 μl of protein A-Sepharose 4B slurry of protein A–Sepharose. Triton X-100 buffer. Protein A–Sepharose was removed, and the supernatant was reacted with the appropriate immune serum for 30 min at 0°C. New protein A-Sepharose slurry was added, incubation continued for 30 min and the beads were washed three times with 1 ml of Triton X-100 buffer, once with the same buffer containing 0.5 M NaCl and finally once with 1 ml of 20 mM Tris–HCl (pH 7.5). In the experiment described in Figure 6, immunoprecipitates were washed three times with 0.3% Triton X-100, 0.1% SDS, 50 mM NaCl, 20 mM Tris–HCl (pH 8.0).

Isolation of Glycoproteins and Labeled Micosomes from Raji Cells

Raji cells labeled for 10 min with ³⁵S-methionine were solubilized in Triton X-100 buffer, particulate material was removed by centrifugation and the supernatant was applied to a column of Sepharose 4B coupled with lens culinaris hemagglutinin (Pharmacia). Bound glycoproteins were desorbed by including 10% a-ethyl-mannoside in the buffer (Cullen and Schwartz, 1978).

To isolate microsomes, labeled Raji cells were washed once in ice-cold hypotonic buffer (10 mM Tris–HCl [pH 7.4], 10 mM KCl and 5 mM MgCl₂) and then broken by gentle homogenization. Nuclei and mitochondria were removed by centrifugation at 5000 × g for 10 min. Microsomes present in the supernatant were pelleted by centrifugation at 20,000 × g for 20 min. Microsomal antigens were solubilized in Triton X-100 buffer. When the membrane disposition of HLA-DR antigens was determined, the postmitochondrial supernatant was incubated for 60 min at 0°C with 0.2 mg/ml proteinase K (Merck). Proteinase action was stopped with 40 μg/ml PMSF, and antigens were solubilized in Triton X-100 buffer.

Detection of Cell-Surface HLA-DR Antigens

To characterize the HLA-DR antigens present on the cell surface, cells were pulse-labeled for 10 min with ³⁵S-methionine and chased for 90 min. After being washed once with Tris–saline buffer, cells were resuspended in the same buffer and incubated at 0°C with an excess of the rabbit anti-DR antisera for 30 min. After the incubation period, the cells were washed twice with Tris–saline buffer to remove unbound antibodies and then lysed into 400 μl of a detergent-containing cell lysis prepared from unlabeled Raji cells. The ratio of labeled to unlabeled cells was 1:5. This means that eventually free antibodies would preferentially recognize unlabeled intracellular forms of antigens (Vitetta and Uhr, 1976). After 30 min of solubilization, particulate material was removed by centrifugation, and the antigen–antibody complexes in the supernatant were recovered by protein A–Sepharose.

Raji mRNA Purification and In Vitro Translation

Messenger RNA from Raji cells was isolated and fractionated as described for SL2 cell mRNA (Dobberstein et al., 1978). Total nucleic acids were extracted from Raji cells by phenol–chloroform isomyl alcohol. Poly(A)-containing RNA (mRNA) was isolated by affinity
chromatography on oligod(T)-cellulose columns. Messenger RNA was size-fractionated on a 10%–30% sucrose gradient. Centrifugation was for 16 hr at 20°C in an Beckman SW40 rotor. For the determination of S values, mouse 28S and 18S rRNA and E. coli 23S and 16S rRNA were run on parallel gradients.

Total or fractionated mRNA was translated in a cell-free system derived from rabbit reticulocytes, (Peltom and Jackson, 1976). Usually, 250 ng of mRNA was translated in a final volume of 25 µl containing 25 µCi 35S-methionine (500 Ci/m mole). The reticulocyte lysate was supplemented with microsomes derived from dog pancreas (0.2 A260 units per 25 µl incubation). Microsomes were prepared and treated as described previously (Garoff et al., 1978; Scheele et al., 1978). Incubation was for 60 min at 37°C. To isolate the microsomes after translation, the cell-free system was brought to 0.4 M KCl and 10 mM EDTA. The microsomes were sedimented through a 10% sucrose cushion by centrifugation at 100,000 × g for 10 min in a Beckman Airfuge. The pellet was treated with Triton X-100 buffer, and solubilized antigens were subjected to immunoprecipitation.

SDS-Polyacrylamide Gel Electrophoresis

Proteins translated in the in vitro cell-free system were characterized by treating a 1 µl aliquot with 25 µl of sample buffer (0.1 M Tris-HCl [pH 6.8], 2% SDS, 1 mM EDTA, 50 mM methionine, 15% sucrose, 10 mM dithiothreitol and bromophenol blue). In vivo labeled microsomal antigens were characterized in a similar way by diluting 1–5 µl of the solubilized antigens with 24–20 µl of the sample buffer. After immunoprecipitation, the antigen–antibody complexes bound to protein A–Sepharose were solubilized in 25 µl of sample buffer. All samples were heated to 95°C for 2 min, cooled to room temperature and alkylated in 50 mM iodoacetamide for 20 min in the dark before being applied to the polyacrylamide gel. The gels consisted of 10%–15% polyacrylamide slabs and were run as described (Blobel and Dobberstein, 1975). The gels were fixed in 10% trichloracetic acid for 30 min and then treated with ENHANCE (New England Nucleon). Gels were dried, and protein bands containing radioactivity were detected by fluorography (Bonner and Laskey, 1974). For quantitation of the protein bands, fluorographs were scanned by the use of a Joyce-Loebel densitometer, and radioactivity was determined as the area under each peak. This is expressed in arbitrary units (AU) in figures. Molecular weight standards were 125I-labeled phosphorylase (90,000), bovine serum albumin (67,000), ovalbumin (46,000), carbon anhydride (28,000) and cytochrome c (14,000).

Materials

RPMI-1640 and Dulbecco’s modified Eagle’s media were from Gibco; 35S-methionine, ENHANCE, the rabbit reticulocyte lysate and the molecular weight standards were from New England Nucleon; oligod(T) cellulose type 3 was from Collaborative Research; protein A–Sepharose CL-4B was from Pharmacia. Cycloheximide was from Boehringer Mannheim; α-methyl-mannoside was from Calbiochem.

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