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Cell-Free Synthesis and Membrane Insertion of Mouse H-2D^d Histocompatibility Antigen and β_2-Microglobulin

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Summary

Messenger RNA from SL2 lymphoma cells was translated in a cell-free system in the presence of microsomal membranes. Mouse H-2D^d histocompatibility antigen was correctly assembled in the microsomal membranes, and transmembrane insertion of the nascent chain was accompanied by glycosylation and cleavage of the signal sequence. H-2K^d antigens, synthesized in vivo, comprised a transmembrane glycoprotein and an unglycosylated protein in the cytoplasm. The glycosylated forms of the H-2D^d and H-2K^d antigens were modified during intracellular transport from the endoplasmic reticulum to the cell surface. β_2-Microglobulin was also synthesized in vitro, and transfer of this protein into microsomal vesicles was accompanied by cleavage of its signal sequence. In the endoplasmic reticulum, β_2-microglobulin can bind to newly synthesized H-2^d glycoproteins. The mRNAs coding for β_2-microglobulin and H-2D^d antigen could be separated on aqueous sucrose gradients.

Introduction

Plasma membranes of eucaryotic cells perform a unique set of functions determined by the number and type of proteins found in the membrane. Intracellular membranes with different functions have different sets of proteins. The question then arises as to how these proteins are synthesized and inserted into the correct membrane. Many plasma membrane proteins, particularly those that span the lipid bilayer, are thought to be synthesized on ribosomes bound to the endoplasmic reticulum and are then assembled in this membrane (Dallner, Siekevitz and Palade, 1966; Morrison and Lodish, 1975). Secretory proteins are also synthesized at this site and transferred across the membrane (Palade, 1975). Both the secretory proteins and the plasma membrane proteins must then be transported to the plasma membrane. At some stage, they must be separated from those proteins destined for other cellular compartments. An understanding of this process of membrane assembly and intracellular transport is therefore crucial to our understanding of the mechanisms by which individual membranes are constructed.

Though details of the transport process are still obscure, great progress has been made in recent years in elucidating the steps by which membrane proteins and secretory proteins become inserted into the membrane of the endoplasmic reticulum. This has been brought about largely by using cell-free systems in which proteins are synthesized in the presence of rough microsomal vesicles. The synthesis of many secretory proteins and some viral membrane glycoproteins has been studied using this technique (Blobel and Dobberstein, 1975a, 1975b; Szczesna and Boime, 1976; Rothman and Lodish, 1977; Toneguzzo and Ghosh, 1977; Katz et al., 1977a; Garoff, Simons and Dobberstein, 1978; Bonatti, Cancedda and Blobel, 1979). In each case, the cell-free system accurately reproduced those features of the cell that lead to insertion of protein into the endoplasmic reticulum. Secretory proteins were transferred across the membrane, where they were inaccessible to added protease (Blobel and Dobberstein, 1975; Szczesna and Boime, 1976); viral membrane glycoproteins were inserted with the correct orientation and glycosylated on the noncytoplasmic side of the membrane (Toneguzzo and Ghosh, 1977; Katz et al., 1977; Garoff, Simons and Dobberstein, 1978; Bonatti, Cancedda and Blobel, 1979), and protease treatment then removed only that portion of the viral protein present on the cytoplasmic side. In most cases, the synthesis of these proteins was preceded by a short amino-terminal extension termed a signal sequence (Milstein et al., 1972; Schechter et al., 1974; Blobel and Dobberstein, 1975a; Campbell and Blobel, 1976; Palmiter, Gagnon and Walsh, 1978; Lingappa et al., 1978). This is thought to locate the ribosomal complex on the membrane so that further synthesis of the nascent polypeptide chain is coupled to membrane insertion. The signal sequence is cleaved during or shortly after transfer.

We have chosen to study the membrane assembly of the major histocompatibility antigens and β_2-microglobulin as a first step toward understanding the intracellular transport and surface expression of this important class of plasma membrane proteins (Klein, 1975; Ivanyi, 1978; Crumpton et al., 1978). The genes coding for these surface proteins are located at opposite ends of the major histocompatibility complex (Nathenson and Cullen, 1974; Bodmer et al., 1978). The genes are extremely polymorphic, so that the antigens expressed on the cell surface differ considerably among individuals. These histocompatibility antigens are the principal targets for graft rejection and are intimately involved in the cellular immune response to viral antigens and modified cell surface antigens. In man they are termed the HLA antigens (Crumpton et al., 1978); in mouse they are the H-2 antigens which comprise the gene products of the H-2D, H-2K and H-2L locus (Klein, 1975; Demant et al.,

1978). They can be isolated from cells treated with detergent using specific alloantisera and have molecular weights ranging from 43,000 to 48,000 (Nathenson and Cullen, 1974). They are tightly associated with β_2-microglobulin, which is a secretory protein with a molecular weight of 12,000 (Rask, Lindblom and Peterson, 1974; Appella et al., 1976). Cells treated with papain release a water-soluble form of the histocompatibility antigens which is still glycosylated and bound to β_2-microglobulin. It contains the amino terminus and leaves a small (6000–7000 dalton) fragment in the plasma membrane (Yamane and Nathenson, 1970; Peterson, Rask and Lindblom, 1974; Ewenstein et al., 1976; Henning et al., 1976). The major part of these antigens is thus located on the noncytoplasmic side of the membrane and is anchored to the plasma membrane by a small hydrophobic segment (Ewenstein et al., 1976; Henning et al., 1976). For HLA antigens, though not for H-2, it is known that this segment spans the lipid bilayer (Walsh and Crumpton, 1977).

Little is known about the biosynthesis of these membrane proteins, though there is evidence to suggest that they are synthesized at an intracellular site before transfer to the plasma membrane (Vitetta and Uhr, 1975). Here we report studies in a cell-free system and in lymphoma cells that allow a detailed description of the events leading to the assembly of mouse H-2^d antigens and β_2-microglobulin in microsomal membranes.

Results

Transfer of β_2-Microglobulin into Microsomal Vesicles

Messenger RNA was isolated from SL2 lymphoma cells which express relatively large amounts of H-2D^d and H-2K^d antigens on their surface membranes (Robinson and Schirrmacher, 1979). It was translated in a cell-free system derived from rabbit reticulocytes in the presence or absence of dog pancreatic microsomes. The products had molecular weights as high as 100,000 and were very similar to those synthesized by SL2 cells during a short 5 min incubation with ^{35}S-methionine (Figure 1; compare lane 1 with lanes 3 and 4). The mRNA had therefore been isolated in an intact state and was efficiently translated by the reticulocyte lysate system. In the absence of added mRNA, the amount of protein synthesized was reduced by more than 85%, and globin was the main product (Figure 1, lane 2). This was derived from residual globin mRNA present in the reticulocyte lysate (Pelham and Jackson, 1976).

The products synthesized in the presence of microsomal membranes were treated with antibodies to β_2-microglobulin. The antigen-antibody complexes were then bound to protein A-Sepharose and washed extensively before application to the gel. The results

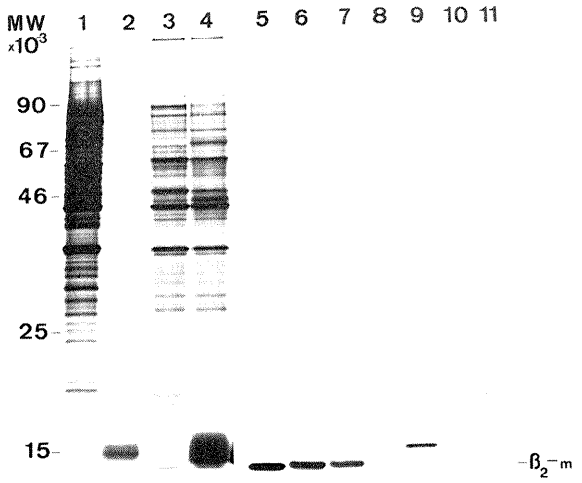

Figure 1. Transfer of β_2-Microglobulin into Microsomal Vesicles

Polypeptides from SL2 lymphoma cells labeled for 5 min with ^{35}S-methionine were separated by SDS-polyacrylamide gel electrophoresis (lane 1). Total polypeptides synthesized in a cell-free system derived from rabbit reticulocytes in the absence (lane 2) or presence (lane 3) of SL2 mRNA and in the presence of SL2 mRNA and microsomal membranes (lane 4) are shown. Rabbit anti-mouse β_2-microglobulin serum was used to precipitate antigens from labeled SL2 cells (lane 6) or from the cell-free system with SL2 mRNA in the presence (lane 5) or absence (lane 9) of microsomal membranes. Two samples with microsomes present during translation were treated with Proteinase K in the absence (lane 7) or presence (lane 8) of NP40. As a control, rabbit nonimmune serum was used to precipitate cell-free products synthesized in the absence (lane 10) or presence (lane 11) of microsomal membranes.

(Figure 1, lane 5) revealed a single protein band with an approximate molecular weight of 12,000, which co-migrated with authentic mouse β_2-microglobulin (Figure 1, lane 6). If the translation products were treated with Proteinase K before immunoprecipitation, β_2-microglobulin was found to be resistant to digestion (Figure 1, lane 7). If membranes were first solubilized with detergent and then treated with protease, the protein was completely degraded (Figure 1, lane 8). β_2-Microglobulin can be transferred in vitro into microsomal vesicles, where it is resistant to protease attack.

When SL2 cell mRNA was translated in the absence of membranes and the products were treated with antibodies to β_2-microglobulin, a major protein with an apparent molecular weight of 15,000 was immunoprecipitated (Figure 1, lane 9), though less readily than authentic β_2-microglobulin (Figure 1; compare lanes 5 and 9). If membranes were added after translation was completed, the same product was obtained, and this was still sensitive to added protease (data not shown). This product could not therefore be transferred into microsomal vesicles after synthesis.

All but one of the secretory proteins that have so far been synthesized in vitro in the absence of membranes have yielded a polypeptide with a higher mo-

lecular weight than the authentic protein (Campbell and Blobel, 1976; Palmiter, Gagnon and Walsh, 1978). The amino-terminal extensions responsible for these higher molecular weights have been termed signal sequences, and they are thought to locate the ribosomal complex on the endoplasmic reticulum membrane during protein synthesis (Blobel and Dobberstein, 1975a). The results presented here suggest that the 15,000 dalton protein synthesized in the absence of membranes is the precursor of β_2-microglobulin (pre-β_2-microglobulin) and contains the signal sequence of this secretory protein. Further work will be needed, however, to show that the difference in molecular weight is due to an extension at the amino terminus. The presence of such an extension in the precursor might be expected to yield a molecule with different antigenic properties than the authentic β_2-microglobulin (Duguid, Steiner and Chick, 1976); this would then explain why the precursor is less readily precipitated by the antibody than is authentic β_2-microglobulin.

Synthesis and Glycosylation of H-2^d Antigens

We compared H-2^d antigens synthesized during a 5 min incubation of mouse SL2 lymphoma cells with ^{35}S-methionine to those synthesized in a cell-free system in the presence or absence of microsomal membranes. The products of the 5 min labeling in vivo were precipitated specifically with alloantisera against either H-2D^d or H-2K^d antigens. The major polypeptide precipitated by anti-H-2D^d serum had an apparent molecular weight of 43,000 (Figure 2, lane 1) and was glycosylated, since it bound to lentil lectin (Figure 2, lane 2). The anti-H-2K^d serum precipitated two major polypeptides (Figure 2, lane 3): one with a molecular weight of 46,000, which was glycosylated, and the other with a molecular weight of 43,000, which was not glycosylated (see Figure 2, lane 4). This latter polypeptide had a molecular weight similar to that of the H-2D^d glycoprotein, but could readily be distinguished from it by using lentil lectin.

The products of the cell-free system in the presence or absence of microsomal membranes were precipitated with the same alloantisera. In the presence of microsomal membranes, the major protein band precipitated by H-2D^d alloantiserum co-migrated with that synthesized in vivo (Figure 2, compare lanes 1 and 6). This strongly suggests that the cell-free system is reproducing those features of the cell necessary for the correct synthesis of the H-2D^d antigen. In contrast, no specific band was precipitated with H-2K^d alloantiserum (Figure 2, lane 8). Both alloantisera also failed to precipitate specific products from the cell-free system in the absence of microsomal membranes (Figure 2, lanes 7 and 9). The alloantisera used in the experiments are very sensitive to the conformation of the H-2 antigen they precipitate (Nathenson and Cullen, 1974; Kvist et al., 1977). A change in conformation

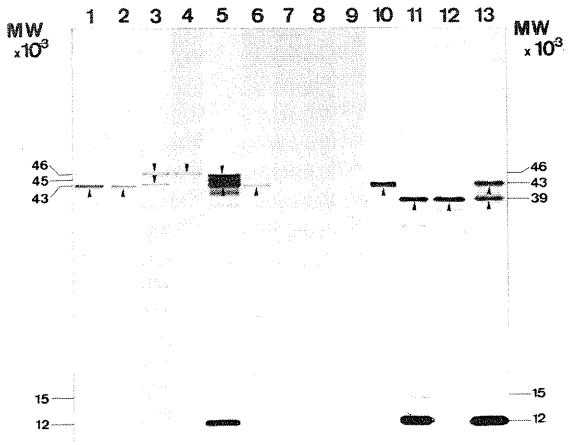

Figure 2. Synthesis and Glycosylation of H-2^d Antigens

H-2^d antigens synthesized in SL2 cells after 5 min labeling with ^{35}S-methionine were either immunoprecipitated with anti-H-2D^d serum (lane 1), anti-H-2K^d serum (lane 3) or rabbit anti-H-2^d serum (lane 5), or treated with lentil lectin-Sepharose, eluted with α-methyl mannoside and then immunoprecipitated with anti-H-2D^d serum (lane 2) or anti-H-2K^d serum (lane 4). H-2^d antigens synthesized in vitro in the presence of microsomal membranes using SL2 mRNA were either precipitated with anti-H-2D^d serum (lane 6), anti-H-2K^d serum (lane 8) or rabbit anti-H-2^d serum (lane 13), or treated with lentil lectin and the bound (lane 10) and unbound (lane 11) fractions immunoprecipitated with rabbit anti-H-2^d serum. The H-2^d antigens synthesized in vitro in the absence of membranes were immunoprecipitated with anti-H-2D^d serum (lane 7), anti-H-2K^d serum (lane 9) or rabbit anti-H-2^d serum (lane 12). Arrows pointing upward indicate H-2D^d antigens; arrows pointing downward indicate H-2K^d antigens. Lanes 1–5, 12 and 13 represent 7 day exposures; the other lanes represent 30 day exposures.

may lead to a drastic reduction in, or complete loss of, the antigenicity. The inability of the H-2 alloantisera to precipitate specific products in some of the experiments in vitro may therefore reflect such changes in conformation. The products could have been synthesized in vitro but not precipitated. To test this possibility, we used a rabbit anti-H-2^d serum that was relatively insensitive to the conformation of the antigen (Kvist, Östberg and Peterson, 1978). When SL2 cells were labeled for 5 min in vivo and the products were treated with this serum, the H-2K^d and H-2D^d antigens, β_2-microglobulin and a protein band of 45,000 daltons were precipitated (Figure 2, lane 5).

To determine whether the H-2K^d antigens are synthesized in vitro in the presence of membranes, we first treated the cell-free products with lentil lectin to isolate the H-2D^d and H-2K^d glycoproteins. This allowed us to distinguish between the H-2D^d glycoprotein and the unglycosylated H-2K^d antigen, which had the same apparent molecular weight. The glycoproteins were eluted and treated with the rabbit anti-H-2^d serum. One major band was precipitated which co-migrated with the H-2D^d glycoprotein (Figure 2; compare lanes 2 and 10). The band co-migrating with the H-2K^d glycoprotein was extremely faint, showing that

the protein is synthesized only in very small amounts in this cell-free system. No band corresponding to the 45,000 dalton glycoprotein was observed. The unglycosylated H-2K^d antigen was also not synthesized in vitro, since no band of the appropriate molecular weight could be precipitated from those polypeptides which did not bind to lentil lectin (Figure 2, lane 11). Since H-2K^d antigen and the 45,000 dalton protein are not synthesized to a significant extent in our cell-free system, the rabbit anti-H-2^d serum could be used to study the synthesis of the H-2D^d antigen in vitro in those cases where the alloantiserum failed to precipitate the antigen. When this serum was used to precipitate products from the cell-free system in the absence of membranes, a form of the H-2D^d antigen which had an apparent molecular weight of 39,000 was obtained (Figure 2, lane 12). The same product was obtained when membranes were added after translation (data not shown). When membranes were present during translation, the 43,000 dalton form of the H-2D^d antigen appeared and the amount of 39,000 dalton form was reduced (Figure 2, lane 13). Since the 39,000 dalton form of the H-2D^d antigen did not bind to lentil lectin (Figure 2, lane 11), it was not glycosylated. These results suggest that the 39,000 dalton form is the unglycosylated precursor of that which is glycosylated in the presence of membranes. Conversion of the nascent precursor to the glycosylated form demands that membranes be present during translation.

Transmembrane Insertion of H-2^d Glycoproteins

H-2^d antigens synthesized in vivo and in vitro were shown to be associated with microsomal membranes, and their disposition across the membrane was determined. Microsomes were prepared from SL2 lymphoma cells that had been labeled for 5 min with ^{35}S-methionine. Labeled H-2^d antigens found in whole cells were recovered in this microsomal fraction (compare Figure 2, lanes 1–5 with Figure 3, lanes 1–3), with the exception of the unglycosylated H-2K^d antigen. Similar experiments were carried out in vitro using fractionated SL2 cell mRNA, which is enriched in mRNA for H-2^d antigens (see below). After translation in the cell-free system, microsomes were separated from the supernatant, and both were treated with the rabbit anti-H-2^d serum. Glycosylated H-2D^d antigen was found only in the membrane fraction, while the unglycosylated precursor was found only in the supernatant (Figure 3, lanes 4 and 5). Thus all glycosylated H-2D^d antigens synthesized in vivo and in vitro were found to be associated with microsomal membranes. If the cell-free system containing microsomal membranes was treated with protease and then with alloantiserum against H-2D^d antigen or rabbit anti-H-2^d serum, the major polypeptide precipitated had a molecular weight of 40,000 (Figure 3, lanes 9 and 10). This was about 3000 daltons smaller than

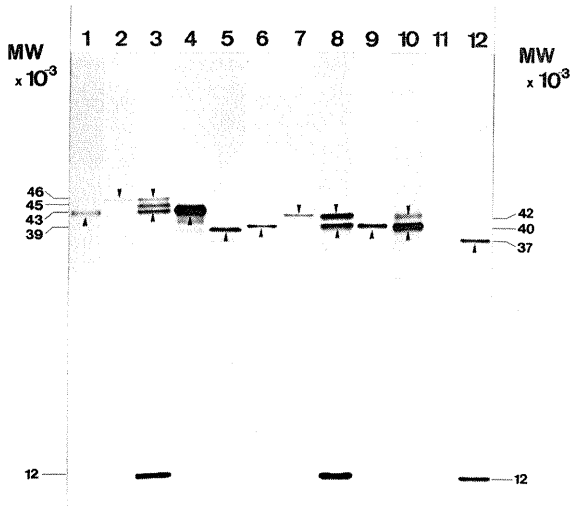

Figure 3. Transmembrane Insertion of H-2^d Glycoproteins

Crude microsomes from SL2 cells labeled for 5 min with ^{35}S-methionine were solubilized in NP40 buffer, and the antigens were precipitated with anti-H-2D^d serum (lane 1), anti-H-2K^d serum (lane 2) or rabbit anti-H-2^d serum (lane 3). For assays using the cell-free system, fractionated mRNA (16–20S) was used, and the antigens were immunoprecipitated with rabbit H-2^d serum from pelleted microsomes (lane 4) or from the membrane-free supernatant (lane 5). Microsomes from labeled SL2 cells were treated with proteinase K, and the antigens were precipitated with anti-H-2D^d serum (lane 6), anti-H-2K^d serum (lane 7) or rabbit H-2^d serum (lane 8). A post-mitochondrial supernatant of these cells containing the microsomes was treated with Papain, and the antigens were immunoprecipitated with anti-H-2D^d serum (lane 12). The cell-free products synthesized in the presence of microsomes were either treated with Proteinase K and immunoprecipitated with anti-H-2D^d serum (lane 9) or rabbit anti-H-2^d serum (lane 10), or treated with Proteinase K in the presence of 0.5% Triton X-100 and immunoprecipitated with rabbit anti-H-2^d serum (lane 11). Arrows pointing upward indicate H-2D^d antigens; arrows pointing downward indicate H-2K^d antigens. Lanes 1–3 represent 5 day exposures; lanes 4–8 and 10–12 represent 20 day exposures; lane 9 represents 40 day exposures.

the glycoprotein precipitated in the absence of protease (Figure 3; compare lanes 4 and 9). The same result was obtained when SL2 cells incubated for 5 min with ^{35}S-methionine were broken and then treated with protease and H-2D^d alloantiserum (Figure 3; compare lanes 1 and 6). If H-2K^d alloantiserum was used instead, a single band of approximately 42,000 daltons was obtained, which was about 4000 daltons smaller than that precipitated in the absence of protease treatment (Figure 3; compare lanes 2 and 7). If in any of these experiments the membrane was disrupted by detergent and the system then treated with protease, no protein could be precipitated (for example, see Figure 3, lane 11). Those portions of the H-2D^d and H-2K^d glycoprotein that were resistant to protease were found to bind to lentil lectin (data not shown) and were therefore glycosylated.

The glycosylated portions of the H-2^d glycoproteins are known to be present on the noncytoplasmic side of the membrane (Nathenson and Cullen, 1974).

Treatment with proteases digests a small part of H-2^d glycoproteins, leaving the much larger glycosylated portion protected within the microsomal vesicles. The H-$2D^d$ and H-$2K^d$ glycoproteins therefore span the membrane, and their disposition must be highly asymmetric. Since the protease completely converts the H-2^d glycoproteins made both in vivo and in vitro to a single species of lower molecular weight, all copies of each protein must have the same orientation in the membrane. An attempt was also made to determine the size of that part of the H-$2D^d$ glycoprotein which spans the membrane by using proteases which cleave the protein either on the cytoplasmic side of the membrane or on the noncytoplasmic side. Papain is known to release a water-soluble form of the H-$2D^d$ glycoprotein from the noncytoplasmic side of the membrane (Nathenson and Cullen, 1974). The cleavage is thought to take place near the membrane surface and is not affected if the membrane glycoprotein is first solubilized by detergent (Henning et al., 1976). SL2 cells labeled for 5 min with ^{35}S-methionine were solubilized with detergent and treated with papain. The H-$2D^d$ glycoprotein was then found to be approximately 6000 daltons smaller than the membrane-bound form (Figure 3; compare lanes 1 and 12), suggesting that this is the size of the fragment that anchors the rest of the protein to the membrane. Since protease treatment of microsomes removes a 3000 dalton segment from the cytoplasmic side of the membrane, this leaves a 3000 dalton part to span the membrane. The transmembrane hydrophobic portions of glycophorin and phage M13 glycoprotein have a molecular weight of approximately 2500 and are 20–25 amino acids long (Wickner, 1976; Furthmayr et al., 1978). A segment of this size is sufficient to span the lipid bilayer just once. Our results on the H-$2D^d$ glycoprotein suggest that it also has a single transmembrane segment.

The insertion of H-$2D^d$ glycoprotein into the microsomal membranes was not dependent on the concomitant synthesis of β_2-microglobulin. The mRNAs coding for these two proteins could be separated, and this did not affect the synthesis and membrane insertion of H-$2D^d$ antigen or the transfer of β_2-microglobulin into microsomal vesicles. Dog pancreatic microsomes contain free β_2-microglobulin, however, and it is known that histocompatibility antigens can be formed from subunits derived from different species (Bodmer et al., 1978). We cannot therefore rule out the possibility that dog β_2-microglobulin affects the folding and glycosylation of the nascent H-$2D^d$ polypeptide chain as it emerges on the intravesicular side of the membrane.

The Signal Sequence of H-$2D^d$ Antigen

Signal sequences have been found on precursors of secretory proteins and two membrane proteins (Campbell and Blobel, 1976; Inouye et al., 1977; Lingappa et al., 1978). If the synthesis of the H-$2D^d$ glycoprotein is preceded by the synthesis of its signal sequence, we would expect to find it contained in the precursor synthesized in the absence of membranes in vitro (Blobel and Dobberstein, 1975b; Szczesna and Boime, 1976). When inserted into microsomal vesicles, the signal sequence would be removed, thereby lowering the molecular weight of the polypeptide chain. This effect, however, is masked in the case of the H-$2D^d$ antigen by glycosylation, which increases the molecular weight so that it exceeds that of the unglycosylated precursor made in the absence of membranes. Glycosylation had therefore to be prevented under conditions where the polypeptide chain was still inserted in the membrane and the signal sequence cleaved.

SL2 cells were preincubated with tunicamycin, which prevents glycosylation of proteins but does not affect their insertion into the membrane (Takatsuki, Kohno and Tamura, 1975; Garoff and Schwarz, 1978). The cells were then labeled with ^{35}S-methionine, and the products were immunoprecipitated with H-$2D^d$ alloantiserum. A protein of approximately 38,000 daltons, which did not bind to lentil lectin, was precipitated (Figure 4, lane 2). It was about 5000 daltons smaller than the H-$2D^d$ glycoprotein synthesized in vivo in the absence of tunicamycin (Figure 4, lane 1) and in vitro in the presence of microsomal membranes (Figure 4, lane 4). This unglycosylated protein was 1000–2000 daltons smaller than the protein obtained in vitro in the absence of microsomal membranes (Figure 4, lanes 2 and 3). This difference is probably due to the removal of the signal sequence from unglycosylated H-$2D^d$ antigen inserted into microsomal membranes. This signal sequence would be 10–20 amino acids long, which is comparable to the size of other known signal sequences (Campbell and Blobel, 1976; Inouye et al., 1977; Lingappa et al., 1978). Further analysis of its sequence is needed.

H-2^d Antigens Are Processed during Intracellular Transport

The H-$2D^d$ antigen is inserted in vitro into rough microsomal membranes and glycosylated. This form of the antigen co-migrated with that found in the microsomal fraction during a 5 min incubation of SL2 cells with ^{35}S-methionine. Taken together, these results strongly suggest that the H-$2D^d$ antigen is assembled in the membrane of the rough endoplasmic reticulum. This form, however, is of lower molecular weight than that found on the surface membrane, so it was important to examine the relationship between the microsomal and the surface forms. SL2 cells were pulsed for 5 min with ^{35}S-methionine and then chased for varying times with excess cold methionine. The results (Figure 5, lanes 2–7) show that H-$2D^d$ glycoprotein inserted into the rough endoplasmic reticulum membrane is converted to a higher molecular weight form

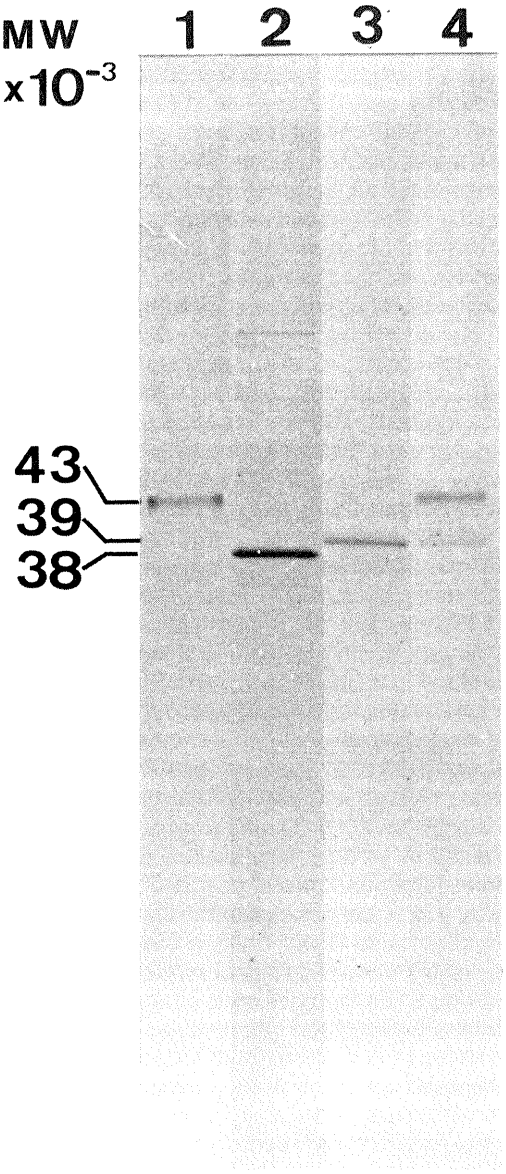

Figure 4. Signal Sequence of H-2D^d Antigen

SL2 lymphoma cells were incubated for 2 hr in the absence (lane 1) or presence (lane 2) of tunicamycin and labeled for 5 min with ^{35}S-methionine, and the antigens were immunoprecipitated with anti-H-2D^d serum. Antigens from the cell-free system in the absence (lane 3) or presence (lane 4) of microsomal membranes were precipitated with rabbit anti-H-2^d serum.

which co-migrates with that found on the surface membrane (Figure 5; compare lanes 7 and 8). This strongly suggests that the H-2D^d glycoprotein inserted into microsomal vesicles in vitro and synthesized during a 5 min pulse with ^{35}S-methionine in vivo is the precursor of the surface H-2D^d antigen. The conversion is first observed 10 min after the beginning of the chase and probably reflects a change in glycosylation. Similar results have also been obtained for the H-2K^d

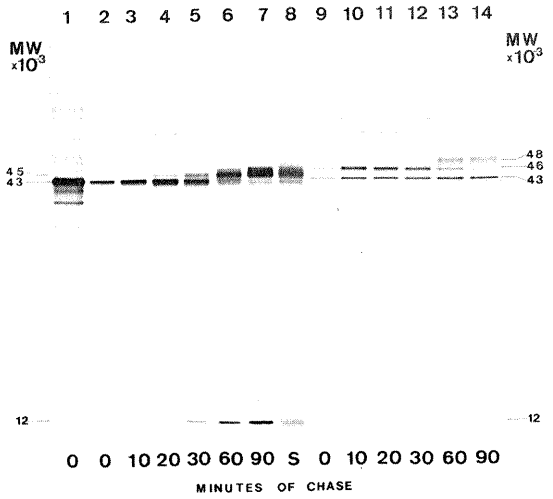

Figure 5. H-2D^d and H-2K^d Antigens Are Processed during Intracellular Transport

SL2 lymphoma cells were pulse-labeled for 5 min with ^{35}S-methionine and chased for the times indicated in the figure by adding excess unlabeled methionine. Antigens were immunoprecipitated with anti-H-2D^d serum (lanes 1–7) or anti-H-2K^d serum (lanes 9–14). ^{125}I-labeled Scell surface antigens (S) were immunoprecipitated with anti-H-2D^d serum (lane 8). Lane 1 is a 20 day exposure; the other lanes are 6 day exposures.

glycoprotein, but not for its unglycosylated form (Figure 5, lanes 9–14). The glycosylated form found in microsomal membranes is converted during the chase into a higher molecular weight protein. The unglycosylated H-2K^d antigen is not converted to a higher molecular weight form, but remains unchanged throughout the period of the chase (Figure 5, lanes 9–14).

Labeled β_2–microglobulin could be precipitated by alloantisera in this pulse-chase experiment immediately after the 5 min pulse (Figure 5, lane 1). Since the alloantisera only precipitate β_2–microglobulin when it is bound to the H-2 antigens, newly synthesized β_2–microglobulin must bind to the H-2 antigens in the rough endoplasmic reticulum. The amount of labeled β_2–microglobulin precipitated with alloantisera increased during the period of chase (Figure 5, lanes 2–7 and 9–14). This is consistent with the theory that there is a pool of unlabeled β_2–microglobulin in the endoplasmic reticulum and that labeled β_2–microglobulin would have to compete for the newly inserted H-2^d antigens. As intracellular transport proceeds during the chase period, labeled β_2–microglobulin remaining in the rough endoplasmic reticulum becomes bound to unlabeled, newly synthesized H-2^d antigens, so that increasing amounts would be precipitated by alloantisera.

Separate mRNAs Code for the H-2D^d Antigen and β_2–Microglobulin

Messenger RNA of SL2 cells was fractionated on an

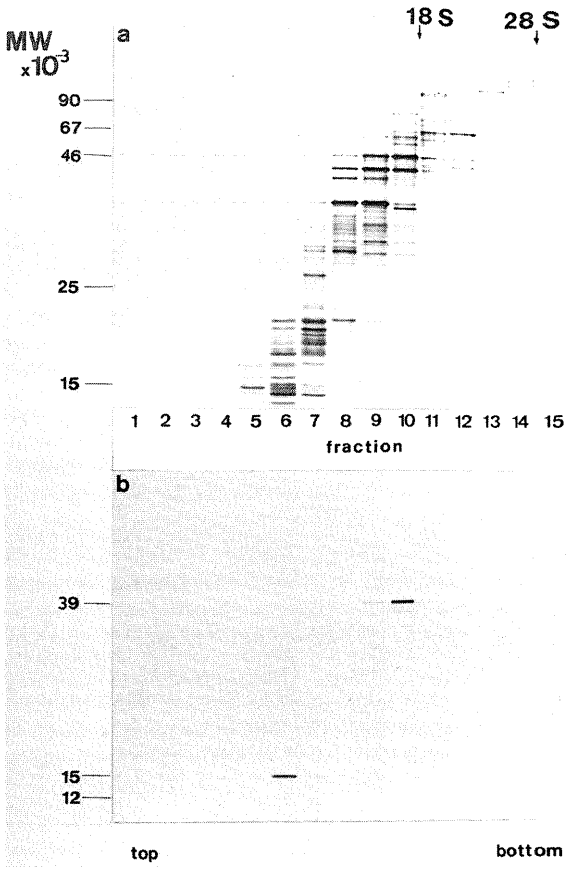

Figure 6. Gradient Centrifugation of SL2 Cell mRNA

SL2 cell mRNA was separated on a sucrose gradient and fractionated, and an equal part of each fraction was translated in the cell-free system. (a) 1 μl aliquots were characterized on an SDS-polyacrylamide gel. (b) From the rest of each fraction, antigens were precipitated with anti-H-2^d serum.

aqueous sucrose gradient. The fractions were translated in vitro and the labeled proteins were characterized on gels. The result shown in Figure 6a indicates that the mRNA species range from 8–30S. The size of the translation products were proportional to the size of the respective mRNA. The translation products were then immunoprecipitated with rabbit anti-H-2^d serum. The result shown in Figure 6b indicates that the mRNA for the H-2D^d antigen and β_2-microglobulin are different and can be separated completely using these sucrose gradients. The estimated sizes of the mRNAs for the H-2D^d antigen and β_2-microglobulin are approximately 17S and 9S, respectively. Other cellular proteins of similar sizes are coded for by mRNAs with similar sedimentation coefficients (Cowan, Secher and Milstein, 1976; Hunter and Garrels, 1977).

Conclusions

Several membrane glycoproteins have been used previously to study membrane assembly in vitro (Rothman and Lodish, 1977; Toneguzzo and Ghosh, 1977; Katz et al., 1977a; Garoff et al., 1978; Bonatti et al., 1979). Viral proteins were chosen for these studies, since the mRNA coding for the membrane proteins constitutes a large proportion of the mRNA in the infected cells. The histocompatibility antigens constitute only a small proportion of the proteins in the plasma membrane of lymphoma cells, and when their total mRNA is translated in vitro, the H-2^d antigens constitute no more than 0.1% of the total proteins synthesized. Nevertheless, it has been possible to study the assembly of these antigens in microsomal vesicles in vitro. This has been possible because the H-2 antisera used have a high specificity and a high affinity for the antigens, and because the cell-free system derived from rabbit reticulocytes (supplemented with pancreatic membranes) accurately reproduced those features of the cell which lead to the assembly of membrane proteins in the endoplasmic reticulum in vivo. It should therefore be possible to study the membrane assembly of any minor membrane component in vitro if antibodies of high specificity and affinity are available.

The surface forms of the H-2^d antigens appear as broad, indistinct bands on SDS-polyacrylamide gels; this probably reflects differences in the amounts of sialic acid bound to each polypeptide chain (Nathenson and Cullen, 1974). In contrast, the microsomal forms of these antigens appear as narrow, sharp bands on SDS gels; this probably reflects the homogeneity of the core oligosaccharides that are coupled to the newly synthesized proteins in the endoplasmic reticulum. This feature of the microsomal forms of these antigens was crucial in determining the sequence of events leading to the assembly of the antigens in microsomal membranes in vitro and in determining the disposition of the proteins across the membrane. Differences in molecular weight as small as 500 daltons could be easily distinguished. This improved resolution also made it possible to distinguish clearly between the products of the H-2D^d and H-2K^d locus, and even to resolve a product that does not originate from either of these loci. This unidentified product had a molecular weight of 45,000 daltons and may represent a product of the H-2L locus (Demant et al., 1978). The H-2D^d alloantiserum precipitated one major glycoprotein with an apparent molecular weight of 43,000, whereas the H-2K^d alloantiserum precipitated two major polypeptide bands of 43,000 and 46,000 daltons; the latter form was glycosylated, while the former was not. The unglycosylated form of the H-2K^d antigen behaved as a cytoplasmic protein and could represent a form of the H-2K^d glycoprotein that failed to become inserted into the membrane of the endoplasmic reticulum. This would mean that the H-2K^d gene product in these lymphoma cells does not compete efficiently with other nascent membrane proteins for those binding sites on the membrane that

mediate protein insertion (Blobel and Dobberstein, 1975a; Warren and Dobberstein, 1978; Kreibich et al., 1978). An alternative possibility is that the cytoplasmic unglycosylated form of the H-2K^d antigen is coded by a different gene than is the glycosylated form. Further work should allow us to distinguish between these two possibilities and should also show whether these two forms are found in normal cells.

There are many similarities between the assembly of the H-2D^d antigen and that of viral membrane glycoproteins. The nascent polypeptide contains a signal sequence, if no membranes are present in the cell-free system, a precursor is synthesized which can no longer be inserted into the membrane. During or shortly after transfer, the signal sequence is cleaved and the portion of the polypeptide on the noncytoplasmic side of the membrane is glycosylated (Katz et al., 1977b; Garoff et al., 1978; Bonatti et al., 1979). Transfer stops when the transmembrane segment of the polypeptide spans the lipid bilayer. For H-2D^d antigen, synthesis of the remainder of the polypeptide results in a 3000 dalton carboxy-terminal segment on the cytoplasmic side of the membrane. The transmembrane segment is about 25 amino acids in length, which is sufficient to span the bilayer once. The major part of the protein is present on the noncytoplasmic side of the membrane. The disposition of this antigen across pancreatic membranes in vitro was identical to that found in microsomes isolated from lymphoma cells.

In contrast to the H-2D^d antigen, very little of the H-2K^d antigen could be synthesized in vitro. The most probable explanations are that the mRNA may be very labile and that certain tRNAs are either not present in the reticulocyte lysate or are not present in sufficient amounts (Revel and Groner, 1978). The disposition of the H-2K^d antigen across the membrane was determined in microsomal vesicles isolated from lymphoma cells and was found to be very similar to that of the H-2D^d antigen.

β_2–Microglobulin could be transferred into microsomal vesicles in vitro, where it was resistant to added protease. Cleavage of its signal sequence occurred during or shortly after transfer, and in the absence of membranes, a precursor was formed which could not then be transferred across the membrane. This protein therefore has all the characteristics of other secretory proteins (Campbell and Blobel, 1976).

The results of studies in vitro and in vivo show that the H-2^d antigens are first assembled in the membrane of the endoplasmic reticulum. Pulse-chase experiments have established that these microsomal forms of the H-2^d antigens are the precursors of those found on the surface membrane having a higher molecular weight. The time course and the magnitude of the increase in molecular weight suggest that a change in glycosylation had occurred in the Golgi complex. The steps in glycosylation of the glycoproteins of vesicular stomatitis virus have been studied in detail (Hunt, Etchison and Summers, 1978; Tabas, Schlesinger and Kornfeld, 1978). "Core" oligosaccharide chains containing a high proportion of mannose residues are transferred as a unit from a lipid intermediate onto the nascent polypeptide chain in the endoplasmic reticulum. During its transport to the plasma membrane, the oligosaccharide chain is modified, and fucose, galactose and sialic acid residues are added to give the complex-type oligosaccharide found on the surface glycoprotein. This change in glycosylation results in a shift to a higher molecular weight which is similar to that observed here for the H-2D^d and H-2K^d glycoproteins, suggesting similar intracellular modifications (Katz et al., 1977b).

β_2–Microglobulin is thought to be needed for the cell surface expression of histocompatibility antigens. Daudi cells, in which the genes for β_2–microglobulin are deleted, do not express HLA antigens on their cell surface even though they possess the genes for these proteins (Bodmer et al., 1978). Our results show that the insertion of H-2 antigens into microsomal membranes in vitro is not dependent on the concomitant synthesis of β_2–microglobulin, but that this protein can bind to newly synthesized H-2^d antigens once it is in the endoplasmic reticulum. If the antigens are inserted into the endoplasmic reticulum membrane in Daudi cells, this would mean that β_2–microglobulin is needed at some stage in intracellular transport.

A greater understanding of the function and cell surface expression of the histocompatibility antigens will be obtained by studying the sequence of the genes and their organization within the major histocompatibility complex. An important step in this direction would involve the molecular cloning of the DNA complementary to the mRNAs for these histocompatibility antigens (Hozumi and Tonegawa, 1976; Breathnach et al., 1978). We have presented here a means of assaying the H-2D^d antigen in a cell-free system and have described a first step in purification of the mRNA, which should help to attain this goal.

Experimental Procedures

Labeling of SL2 Lymphoma Cells

SL2 lymphoma cells were grown in ascites form in DBA/2 mice (Bomholtgård, Denmark) and harvested from the peritoneal cavity 8–10 days after inoculation with 2 × 10^6 SL2 cells. The cells were washed 3 times in methionine-free RPMI medium.

When labeled, newly synthesized proteins in the rough endoplasmic reticulum were required, cells (4 × 10^6 in 200 μl) were incubated at 37°C for 5 min in methionine-free RPMI medium containing 200 μCi ^{35}S–methionine (400–500 Ci mM^{-1}). In the pulse-chase experiments, 4 × 10^6 SL2 cells were labeled for 5 min at 37°C in 200 μl RPMI medium containing 200 μCi ^{35}S–methionine. 20 ml of RPMI medium containing unlabeled methionine and 5% fetal calf serum were added. Aliquots (5 × 10^5 cells) were removed at the times indicated in Figure 5 and treated with antiserum. Cell surface components were labeled with ^{125}I using the lactoperoxidase method, as described by Robinson and Schirrmacher (1979).

Treatment of SL2 Cells with Tunicamycin

SL2 cells (4×10^6 cells in 200 μl) were incubated in methionine-free RPMI medium at 37°C for 2 hr with tunicamycin (2 μg ml^{-1}) (Takatsuki, Kohno and Tamura, 1975) and then labeled for 5 min with ^{35}S-methionine (1 mCi ml^{-1}). Cells were transferred into cold Tris-saline buffer containing 100 μg ml^{-1} cycloheximide and washed once. For immunoprecipitation, cells were solubilized by the addition of NP40 to 1%, the nuclei were pelleted and the supernatant was passed over a lentil lectin-sepharose column. The unbound material was treated with antiserum.

Extraction of RNA

SL2 cells from 20 mice were lysed in 400 ml of 50 mM Tris-HCl (pH 7.5), 0.12 M NaCl, 5 mM EDTA, 2% SDS, and total nucleic acid was extracted with 400 ml phenol:chloroform:isoamyl alcohol (50:50:2). Following centrifugation (5000 × g for 10 min), the aqueous phase was extracted twice with phenol:chloroform:isoamyl alcohol until no protein remained at the interface. Poly(A)-containing RNA was isolated by adsorption at room temperature to oligo(dT)-cellulose in 0.4 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% SDS and elution with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% SDS (Aviv and Leder, 1972).

Sucrose Gradient Centrifugation of mRNA

mRNA from SL2 cells (200 μg) was heated to 70°C for 2 min in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% SDS, cooled rapidly and layered onto a 10–30% sucrose gradient containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.2% SDS. The gradient was centrifuged in a Beckman SW40 rotor at 40,000 rpm for 20 hr at 20°C. For the determination of S values, mouse 28S and 18S rRNA, E. coli 23S and 16S rRNA and tRNA were run on parallel gradients. The gradient containing mRNA was fractionated and the mRNA from each fraction was recovered by ethanol precipitation. After dissolving the pellet in 100 μl of 10 mM Tris-HCl (pH 7.5), the mRNA was translated in the reticulocyte lysate system. 2 μl each were used in a final 25 μl sample volume.

In Vitro Translation

Total SL2 cell mRNA or mRNA fractionated on a sucrose gradient was translated in a cell-free system derived from rabbit reticulocytes (Pelham and Jackson, 1976; Shields and Blobel, 1978) in a final volume of 25 μl using 30 μCi ^{35}S-methionine. Where indicated, 1 μg of SL2 cell mRNA and 0.2 A_{260} units of dog pancreatic microsomes (pretreated with nuclease and EDTA) were added. Microsomes were prepared and treated as described previously (Scheele, Dobberstein and Blobel, 1978; Garoff et al., 1978). Incubation was at 30°C for 90 min.

Isolation of Labeled Microsomes

After translation, the cell-free system was brought to 0.5 M KCl, 10 mM EDTA, and microsomes were sedimented through 10% sucrose by centrifugation at 20,000 × g for 20 min. A membrane-free fraction was obtained by centrifuging the resulting supernatant at 100,000 × g for 30 min.

Microsomes were prepared from SL2 lymphoma cells labeled for 5 min with ^{35}S-methionine following breakage and removal of nuclei and mitochondria by centrifugation at 7000 × g for 10 min. From the resulting post-mitochondrial supernatant, microsomes were pelleted by centrifugation at 20,000 × g for 20 min.

Protease Treatment

After translation, some samples of the cell-free system containing microsomes were treated with Proteinase K (100 μg/ml^{-1}) at 4°C for 90 min (Blobel and Dobberstein, 1975a), and the reaction was terminated by the addition of phenylmethyl sulfonyl fluoride (100 μg/ml^{-1} 7 and serum albumin (10 mg/ml^{-1}). For protease treatment of microsomes from SL2 cells labeled for 5 min with ^{35}S-methionine, a post-mitochondrial supernatant was treated with Proteinase K as above.

Detergent extracts of SL2 cells were treated for 30 min at 37°C with papain (0.5 mg/ml^{-1}) in the presence of 1% NP40 and Cysteine (0.25 mg/ml^{-1}), essentially as described by Henning et al. (1976). Proteolysis was terminated by the addition of neutralized iodoacetic acid to a final concentration of 0.5 mg/ml^{-1}.

Affinity Chromatography and Immunoprecipitation

Glycoproteins were adsorbed to lentil lectin-Sepharose and eluted with α-methyl mannoside as described by Cullen and Schwartz (1976). For immunoprecipitation, an aliquot of the cell-free system (24 μl) was mixed with an equal volume of NP40 buffer [10 mM Tris-HCl (pH 7.5), 1% NP40, 0.15 M NaCl, 2 mM EDTA] and treated with 1 μl of nonimmune serum for 10 min at 25°C and then with 40 μl of a 1:1 slurry of protein A-Sepharose in NP40 buffer for 30 min. The supernatant was recovered and treated with 1 μl of the appropriate immune serum followed by protein A-Sepharose. The beads were washed 3 times with 1 ml NP40 buffer, once with 1 ml of NP40 buffer containing 0.5 M NaCl and once with 1 ml of water. Labeled antigens of SL2 cells were extracted with 1% NP40, and the antigens were immunoprecipitated as described for the cell-free products.

SDS-Polyacrylamide Gel Electrophoresis

The products of cell-free synthesis were characterized by treating a 1 μl aliquot with 25 μl of sample buffer [100 mM Tris-HCl (pH 8.8), 4% SDS, 1 mM EDTA, 50 mM methionine, 15% (w/v) sucrose, 10 mM DTT and bromophenol blue]. When labeled products from SL2 cells were characterized, cells were lysed in 1% NP40, the nuclei were pelleted by centrifugation and the proteins were precipitated from the supernatant with 10% TCA. The resulting pellet was washed with 90% acetone to remove the detergent, and the protein was solubilized in sample buffer as described above. After immunoprecipitation, the antigen-antibody complexes bound to protein A-Sepharose were solubilized in 50 μl of sample buffer.

All samples were heated at 90°C for 2 min, alkylated and applied to a 10–15% polyacrylamide slab gel as described previously (Blobel and Dobberstein, 1975a). After the gel was fixed and dried, labeled protein bands were detected by fluorography (Bonner and Lasky, 1974). Molecular weights were calculated by reference to the mobilities of standard proteins: phosphorylase (90,000), bovine serum albumin (67,000), ovalbumin (46,000), chymotrypsinogen A (25,000) and rabbit globin (15,000).

Source of Antisera

The rabbit anti-mouse β_2-microglobulin serum was provided by E. Appella (National Cancer Institute, Bethesda, Maryland). The anti-H-2D^d antigen serum used was the alloantiserum D4, which detects the private specificity on the H-2D gene product of the H-2^d haplotype. The anti-H-2K^d antigen serum is the alloantiserum D31b which detects the private specificity of the H-2K gene product of the H-2^d haplotype (Klein, 1975). They were supplied by J. G. Ray (Research Resources, NIH, Bethesda, Maryland).

The rabbit anti-H-2^d serum reacts with several H-2^d gene products (H-2D^d, H-2K^d and probably H-2L^d) and β_2-microglobulin (Kvist et al., 1978). It was provided by P. A. Peterson (Uppsala, Sweden).

Materials

Oligo(dT)-cellulose (T3) was from Collaborative Research (Waltham, Massachusetts); rabbit reticulocyte lysate and ^{35}S-methionine were from New England Nuclear (Boston, Massachusetts); Proteinase K was from Merck (Darmstadt); protein A-Sepharose CL-4B was from Pharmacia (Uppsala, Sweden); and RPMI 1640 medium was from GIBCO (Glasgow, Scotland).

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