

Intracellular Transport of Membrane Glycoproteins: Two Closely Related Histocompatibility Antigens Differ in Their Rates of Transit to the Cell Surface

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ABSTRACT The intracellular transport of two closely related membrane glycoproteins was studied in the murine B cell lymphoma line, AKTB-1b. Using pulse-chase radiolabeling, the kinetics of appearance of the class I histocompatibility antigens, H-2K^k and H-2D^k, at the cell surface were compared and found to be remarkably different. Newly synthesized H-2K^k is transported rapidly such that all radiolabeled molecules reach the surface within 1 h. In contrast, the H-2D^k antigen is transported slowly with a half-time of 4–5 h. The rates of surface appearance for the two antigens closely resemble the rates at which their Asn-linked oligosaccharides mature from endoglucosaminidase H (endo H)-sensitive to endo H-resistant forms, a process that occurs in the Golgi apparatus. This suggests that the rate-limiting step in the transport of H-2D^k to the cell surface occurs before the formation of endo H-resistant oligosaccharides in the Golgi apparatus. Subcellular fractionation experiments confirmed this conclusion by identifying the endoplasmic reticulum (ER) as the site where the H-2D^k antigen accumulates. The retention of this glycoprotein in the ER does not appear to be due to a lack of solubility or an inability of the H-2D^k heavy chain to associate with β_2 -microglobulin. Our data is inconsistent with a passive membrane flow mechanism for the intracellular transport of membrane glycoproteins. Rather, it suggests that one or more receptors localized to the ER membrane may mediate the selective transport of membrane glycoproteins out of the ER to the Golgi apparatus. The fact that H-2K^k and H-2D^k are highly homologous ($\geq 80\%$) indicates that this process can be strongly influenced by limited alterations in protein structure.

Proteins synthesized on membrane-bound ribosomes of the rough endoplasmic reticulum (RER)¹ may be directed to a variety of cellular locations such as the endoplasmic reticulum (ER), Golgi apparatus, lysosomes, and plasma membrane, or they may be secreted (1, 2). How these diverse proteins are routed from the RER to their appropriate destinations is poorly understood. A widely held view is that sorting-out signals reside in the polypeptide and/or carbohydrate moieties of newly synthesized proteins that interact with appropriate

receptor molecules to direct them to their particular cellular sites (2).

Recent studies have suggested that interaction with specific receptors may occur in the initial stages of transport of secretory proteins and viral membrane glycoproteins to the cell surface. Lodish and co-workers studied the secretion of five proteins by rat hepatoma cells and demonstrated differences of at least threefold in the rates of secretion (3). The rate-limiting step in the intracellular transit of these proteins was identified as the movement from the RER to the Golgi apparatus. Similarly, Fitting and Kabat observed marked differences in the rates of surface appearance of two viral glycoproteins synthesized by cells infected with murine leu-

¹ *Abbreviations used in this paper:* endo H, endo- β -N-acetylglucosaminidase H; ER, endoplasmic reticulum; RER, rough endoplasmic reticulum.

kemia virus (4). They showed that the rate-limiting step in the transfer of the slower glycoprotein occurred in the RER. Both groups suggested that the selective nature of the transport process was consistent with the presence of one or more membrane-bound receptors in the RER that mediate protein export from this organelle to the Golgi apparatus. Presumably such a receptor would recognize specific structural features of the transported polypeptides. In accordance with this hypothesis are recent reports in which defined alterations in the polypeptide sequences of VSV G glycoprotein (5) and a murine λ_2 immunoglobulin chain (6) either slow or halt their intracellular transport at the level of the ER.

Thus far, studies of this nature have been restricted to secretory proteins and viral membrane glycoproteins. Consequently, we investigated whether such selectivity in the transport process could also be demonstrated for plasma membrane proteins that are synthesized as part of a cell's normal repertoire. We chose as a model system the class I histocompatibility antigens of the mouse, specifically H-2K and H-2D. Both antigens are enriched on the surface of lymphoid cells where they play an important role in immune processes such as the recognition of transformed or virally infected cells (7). Class I antigens consist of a glycosylated heavy chain (M_r 45,000) that is highly polymorphic and a noncovalently associated protein, β_2 -microglobulin (M_r 12,000). Sequence studies have revealed that a high degree of homology exists between the heavy chains of the H-2K and H-2D antigens, typically 75–85% depending on the alleles examined (8). Further, the sites of attachment of their 2–3 Asn-linked oligosaccharides are conserved. The use of this model system has a considerable advantage over those used previously because of the extensive sequence homology of these antigens and the fact that they are co-expressed by a given cell. Therefore, the effects that limited structural alterations have on the transport of membrane glycoproteins to the cell surface can be examined.

MATERIALS AND METHODS

Materials: Lactoperoxidase, glucose oxidase, aprotinin, UDP-*N*-acetylglucosamine, GDP-mannose, CMP-*N*-acetylneuraminic acid, and 5'-AMP were products from the Sigma Chemical Co. (St. Louis, MO). UDP-[6-³H]-glucose (4.7 Ci/mmol), [2-³H]adenosine 5'-monophosphate (13.0 Ci/mmol) [³⁵S]methionine (1,490 Ci/mmol), and carrier-free Na¹²⁵I were obtained from the Amersham Corp. (Arlington Heights, IL). UDP-*N*-acetyl-[6-³H]glucosamine (6.6 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Protein A-Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ) and fixed *Staphylococcus aureus* cells of the Cowan I strain were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Neuraminidase from *Vibrio cholerae* was obtained from Calbiochem-Behring Corp. and *Streptomyces plicatus* endo- β -*N*-acetylglucosaminidase H was the kind gift of Dr. A. Tarentino, N. Y. State Department of Health (Albany, NY).

Cells and Antibodies: The B cell lymphoma AKTB-1b (H-2^k haplotype) was generously provided by Drs. Zatz and Mathieson (9). It was grown in AKR/J mice (Jackson Laboratories, Bar Harbor, ME) by injection of 10⁶ cells into the tail vein. 2 wk after injection, the tumor was isolated as a splenic mass and was processed as described below.

Hybridomas producing the 11-4.1 (anti H-2K^{k,q,r}; reference 10) and the 15-5.5S (anti H-2D^k and H-2K^d; reference 11) monoclonal antibodies were obtained from the Salk Institute (La Jolla, CA) and the American Type Culture Collection (Rockville, MD), respectively. Both antibodies were coupled at a ratio of 2.5 mg/g of CNBr-activated Sepharose 4B as described previously (12). Mouse alloantiserum directed against H-2D^k (E32; [B10.AMx]F₁ anti-B10.BR) was obtained from the National Institutes of Allergy and Infectious Diseases Serum Bank (Bethesda, MD), and alloantiserum specific for H-2K^k (JF-03; A.TL anti-A.AL) was generously provided by Dr. J. Freed, National Jewish Hospital and Research Center (Denver, CO).

Radiolabeling of AKTB-1b Cells: Splens bearing the AKTB-1b tumor were removed aseptically, placed in RPMI 1640, and dissected free of

fat and connective tissue. A single cell suspension was prepared by gently pressing the tissue through a stainless steel screen. The cells were washed twice with the same medium, and erythrocytes were then removed by hypotonic lysis. Microscope examination revealed >95% lymphoblastoid cells (~5 × 10⁸ per spleen) with a viability ranging from 90–98% as determined by dye exclusion. Before radiolabeling, the cells were washed twice with complete phosphate-buffered saline (PBS) containing 5.5 mM glucose.

For pulse-chase studies using [³⁵S]methionine as radiolabel, cells were first suspended in methionine-free RPMI 1640 (2 × 10⁶/ml) and placed in an incubator at 37°C for 20 min. After centrifugation, the cells were resuspended (2.5 × 10⁷/ml) in the same medium containing 0.5 mCi/ml [³⁵S]methionine and incubated at 37°C for 10 min (pulse). The chase was initiated by the addition of 9 vol of RPMI 1640 containing 1.0 mM methionine and 5% fetal bovine serum. After incubation at 37°C for various times, aliquots containing 2 × 10⁶ cells were removed and rapidly chilled by the addition of 4 ml of ice-cold PBS. Radiolabeled antigens were then isolated as described below.

For surface radioiodination of AKTB-1b cells, ~10⁷ cells were subjected to the lactoperoxidase labeling technique (13). Viability remained in excess of 90% throughout the procedure. An additional 2 × 10⁷ nonradioactive cells were added to the labeled cells to serve as a source of carrier proteins during the isolation of ¹²⁵I-labeled antigens (see below).

Lysis of Cells and Isolation of Class I Antigens: Unless otherwise indicated, all procedures were conducted at 4°C. Radiolabeled cells (typically 2 × 10⁶) were washed by centrifugation with 0.75 ml of 0.1 M Tris, pH 7.4 containing 0.05 M NaCl, 1 mM MgCl₂, 10 mM iodoacetamide, and 1% aprotinin. Lysis was accomplished by resuspending the cell pellet in 0.2 ml of the same solution containing 0.5% Nonidet P-40 and vortexing vigorously. After incubation for 20 min, lysates were centrifuged at 17,500 g for an additional 20 min. The supernatant fractions were precleared by the addition of 5 μ l of normal mouse serum and 40 μ l of a 10% suspension of fixed *Staphylococcus aureus* cells. After centrifugation to remove the bacteria, the procedure was repeated one additional time. The precleared lysates were incubated with 40 μ l of a 50% suspension in 0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 0.15 M NaCl (NTS) of either the 11-4.1 or the 15-5.5S immobilized monoclonal antibodies. After constant shaking for 2 h, the Sepharose beads were recovered by centrifugation and washed sequentially with 0.75 ml of the following solutions: 0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 1 mM EDTA (NTE); NTE; NTE containing 0.5 M NaCl; NTE containing 0.15 M NaCl and 0.1% SDS; and 0.1% Nonidet P-40, 10 mM Tris, pH 7.4. Class I antigens were eluted from the beads by heating at 95°C for 4 min in 0.1 ml of SDS PAGE sample buffer and were then analyzed by SDS PAGE. Linear 10–15% polyacrylamide gradients were used, and the concentration of Tris in the stacking and resolving gels was halved relative to the original protocol of Laemmli (14).

In some instances, the isolated antigens were subjected to digestion with endo H before gel electrophoresis. In these cases, antigens were eluted from the Sepharose beads by heating with 0.14 ml of 10 mM Tris, pH 7.6 containing 1% SDS. Bovine serum albumin (20 μ g) was added as carrier, and proteins were precipitated by the addition of 1.0 ml of cold acetone. After standing at –20°C overnight, the precipitates were collected by centrifugation and dissolved by heating (95°C for 4 min) in 30 μ l of 0.1 M sodium citrate, pH 6, containing 0.075% SDS and 0.2% β -mercaptoethanol. Aprotinin was added (1%) and the solution was divided into two equal parts, one of which received 2 mU of endo H, whereas the other served as an undigested control. In addition, ribonuclease B (10 μ g) was typically added as an internal control to assess the completeness of the endo H digestion. Incubations were conducted at 30°C for 15–20 h and were terminated by the addition of SDS PAGE sample buffer.

For experiments involving the detection of class I antigens at the cell surface, an alternative protocol was used based on the method of Ploegh et al. (15). Briefly, aliquots of AKTB-1b cells (2 × 10⁶) obtained from pulse-chase or surface radioiodination experiments were resuspended in 0.1 ml of calcium, magnesium-free phosphate-buffered saline (PBS) + glucose and incubated (4°C for 30 min) with either the anti-H-2K^k (8 μ l) or the anti-H-2D^k (15 μ l) alloantiserum. Previous studies had determined that these conditions were optimal for maximum recovery of surface antigen. The polyclonal antibodies were required in this procedure because the monoclonal antibodies exhibited poor binding to antigen when not immobilized on Sepharose beads. High performance liquid chromatography peptide maps revealed that the monoclonal and polyclonal antibodies recognized identical proteins (not shown). After the incubation, excess antibody was removed by washing the cells twice by centrifugation. Lysis of the cell pellet was accomplished by the addition of 0.2 ml of complete lysis buffer containing 8 × 10⁶ cell equivalents of lysate, prepared from nonradioactive cells. After 20 min on ice, the lysates were clarified by centrifugation (17,500 g for 20 min) and immune complexes were isolated by incubation for 1 h with 50 μ l of a 50% suspension of protein A-Sepharose in NTS. The beads were separated by centrifugation, and the supernatant fractions were removed for subsequent isolation of intracellular antigens (see below). After washing the protein A-Sepharose as described earlier for the immobilized monoclonal antibodies, the cell surface antigens were eluted either with SDS

PAGE sample buffer or with 10 mM Tris, pH 7.6, 1% SDS in preparation for endo H digestion. The supernatant fractions remaining after removal of the protein A-Sepharose were subjected to an additional round of antibody treatment to retrieve those antigens that were not available to the first round (intracellular fraction). This was accomplished as described above for isolation of total cellular antigens commencing with the preclearance of lysates using fixed *S. aureus* cells. The only alteration was an increase in the amount of immobilized monoclonal antibody (50 μ l of a 50% suspension). This amount was well in excess of that required to bind to all of the specific antigens present in 10^7 AKTB-1b cells.

Subcellular Fractionation of Pulse-Chase Labeled AKTB-1b

Cells: Two samples of tumor cells (5×10^7) were subjected to a 10-min pulse with [35 S]methionine using the conditions described earlier. At the end of the pulse, 40 ml of RPMI 1640 containing 1 mM methionine, 5% fetal bovine serum, and 1.5×10^8 unlabeled cells (added as carrier) were added to both samples. The unlabeled cells had previously been subjected to the same manipulations used for the radioactive cells except that no radioisotope was present (mock pulse). One of the two samples was rapidly chilled (pulse) and the other was placed in an incubator for an additional 2.5 h (pulse-chase). To assay the activities of various marker enzymes in subcellular fractions, a third sample was prepared that contained 2×10^8 unlabeled cells also previously subjected to a mock pulse protocol.

The method used for the subcellular fractionation of tumor cells was essentially the same as that described by Goldberg and Kornfeld (16) except that swollen cells (2×10^8) were homogenized in a volume of 2 ml with 40–50 strokes of a tight fitting Dounce homogenizer, and sucrose gradients were prepared in Beckman SW 27.1 tubes (Beckman Instruments, Inc., Palo Alto, CA) by overlaying samples with 1.5 ml of 40%, 2.5 ml of 35, 30, and 25%, and 1.0 ml of 20% (wt/wt) sucrose all in 1 mM Tris, pH 8.0; 1 mM EDTA (17). After centrifugation at 25,000 rpm for 15 h, 22 fractions (0.5 ml) were collected from the top by means of an automated gradient sampler followed by two fractions of 1.0 ml. For the radioactive pulse and pulse-chase samples, these fractions were used directly for immunoprecipitation of H-2K^k and H-2D^k (see below). For the unlabeled sample, each fraction was diluted to 5 ml with Tris-EDTA and centrifuged at 45,000 rpm for 50 min in a Beckman Ti.50 rotor. Pellets were resuspended in 40 μ l of water by repeated passage through a plastic pipet tip and assayed for enzymatic activities.

For the isolation of radiolabeled class I antigens from gradient fractions, the membranes of each fraction were first solubilized by the addition of 1/10 vol of ten times concentrated lysis buffer. After incubating for 30 min, the fractions were precleared twice as outlined earlier except that the bacteria were sedimented at 17,000 *g* for 30 min. Antigens were isolated sequentially by the addition of 60 μ l of a 50% suspension of immobilized monoclonal antibody followed by continuous agitation for 3 h. The Sepharose beads were washed in the usual manner, and antigens were eluted and analyzed by SDS PAGE.

RESULTS

To study the intracellular transport of closely related cell surface glycoproteins in a single cell type, a murine B cell lymphoma line was selected that expresses class I histocompatibility antigens on its surface. AKTB-1b cells were pulse-labeled with [35 S]methionine and chased for various lengths of time. The H-2K^k and H-2D^k antigens were isolated using specific antibodies and analyzed by SDS PAGE. Initially, the enzyme endo H was used to assess the time required for the newly synthesized antigens to acquire complex oligosaccharides, a process that occurs in the Golgi apparatus (18). Under appropriate conditions, this approach can provide an estimate of the rates at which H-2K^k and H-2D^k are transferred from the RER to the Golgi apparatus. Subsequently, the analysis was extended by studying the kinetics of surface appearance of the two antigens as well as their detailed subcellular distributions at different times after their synthesis.

Analysis of Whole Cell Lysates

AKTB-1b cells were pulsed for 10 min with [35 S]methionine and lysed after various periods of chase lasting up to 2 h. Fig. 1a shows the SDS PAGE analysis of the H-2K^k antigen isolated from the lysates. Two prominent bands are evident immediately after the pulse (lane 1). The slower migrating species corresponds to the H-2K^k heavy chain (M_r 45,000),

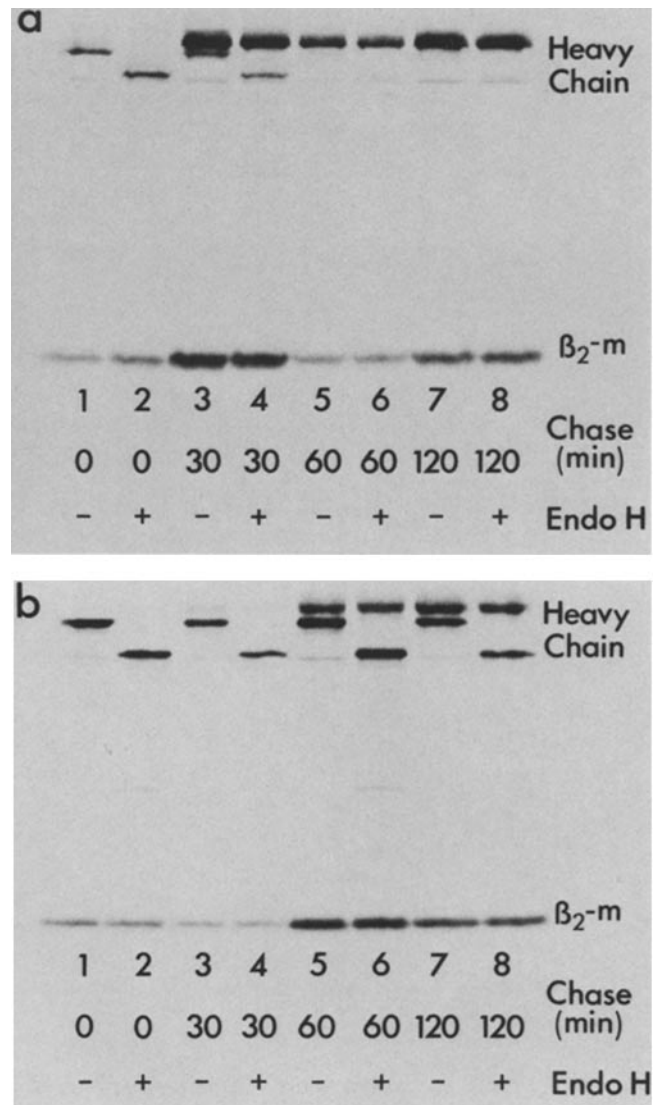


FIGURE 1 Endo H digestion of pulse-chase labeled H-2K^k and H-2D^k antigens. Antigens were isolated from lysates of whole cells at various times after 10-min pulse with [35 S]methionine. One-half of the immunoprecipitates was digested with endo H, whereas the other half was mock digested. The digests were subjected to SDS PAGE using 10–15% gradient gels, and radioactive proteins were detected by fluorography. (a) Analysis of the H-2K^k antigen. (b) Analysis of the H-2D^k antigen. Some variability in the recovery of the antigens was encountered in this experiment resulting in different band intensities in the various lanes. β_2 -m, β_2 -microglobulin.

whereas the species of higher mobility corresponds to β_2 -microglobulin (M_r 12,000; reference 8). In addition, a minor band migrating slightly ahead of the heavy chain can be observed in all of the lanes. This is probably actin, a common component in lymphoid tissues (19). Digestion of the pulse-labeled antigen with endo H causes an increase in mobility of the heavy chain (lane 2). Endo H cleaves between the two GlcNAc residues that are present in the core region of high mannose type oligosaccharides, leaving only a single GlcNAc residue attached to the polypeptide chain (20). Therefore, all H-2K^k molecules labeled in a 10-min pulse contain high mannose oligosaccharides. No effect of the enzyme treatment is observed on β_2 -microglobulin which is not glycosylated. Chasing the cells with an excess of unlabeled methionine results in a rapid time-dependent decrease in the mobility of the heavy chain (compare lanes 1 and 3). That this decrease

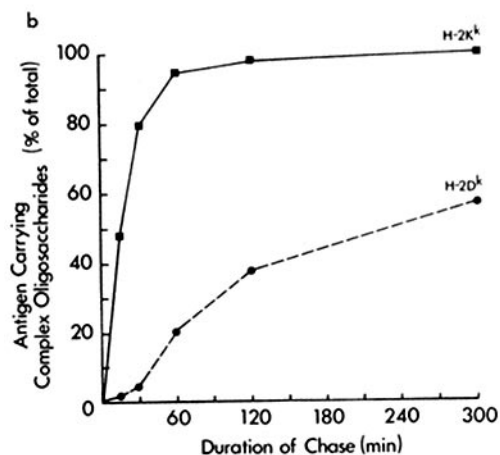
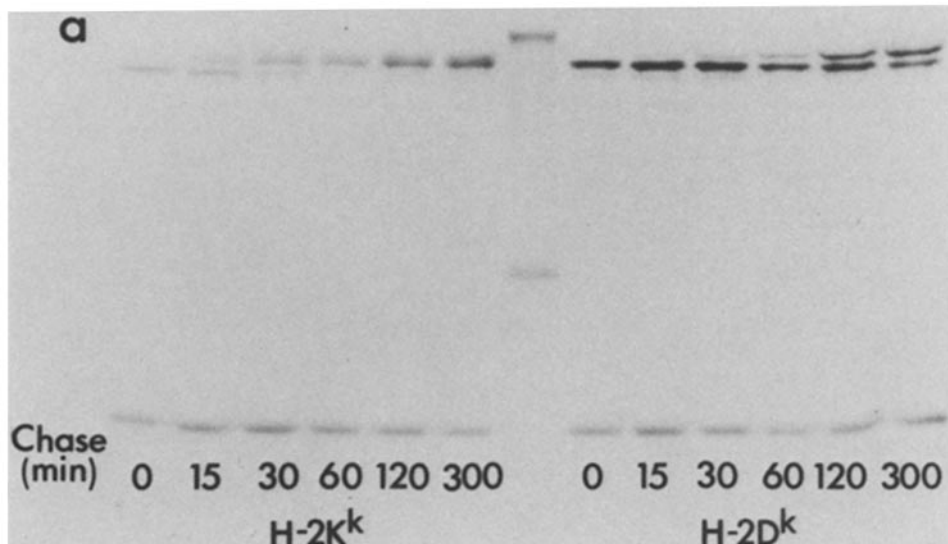


FIGURE 2 Quantitative analysis of the kinetics of oligosaccharide processing for H-2K^k and H-2D^k. Antigens were isolated at various chase times from whole cell lysates and subjected to SDS PAGE (a). Murine IgG was included in the center lane as a molecular weight standard (heavy chain, M_r 53,000; light chain, M_r 23,000). To quantitate the relative amounts of antigen that possess either high mannose or complex oligosaccharides, the intensities of the corresponding H-2K^k or H-2D^k heavy chain bands were determined using an LKB Ultrascan laser densitometer. The percentage of total antigen that contains complex oligosaccharides was calculated and is presented as a function of the duration of chase (b). The slower electrophoretic mobility of the H-2D^k heavy chain at all chase times relative to that of H-2K^k arises from the fact that the former polypeptide possesses three N-linked oligosaccharides, whereas the latter has two (21).

in mobility is due to processing of the oligosaccharide chains to the complex form was confirmed by digestion with endo H. The more slowly migrating species is clearly resistant to the action of the enzyme (lanes 4, 6, and 8). These data indicate that the oligosaccharide chains of the H-2K^k heavy chain are processed to the complex form quite rapidly; essentially all of the molecules are endo H-resistant within 60 min after synthesis.²

Analysis of the H-2D^k antigen isolated from the same lysates revealed markedly different kinetics when compared to H-2K^k. As shown in Fig. 1b, this antigen is also completely sensitive to the action of endo H immediately after the pulse as observed for H-2K^k (lanes 1 and 2). However, after 60 min of chase (the time at which all of H-2K^k is endo H-resistant), greater than half of the H-2D^k molecules are still sensitive to digestion (lanes 5 and 6). In fact, even after 2 h of chase, a substantial population of the H-2D^k molecules remains endo H-sensitive.

This difference between the two antigens in the kinetics of acquisition of endo H resistance was studied in greater detail as illustrated in Fig. 2a. In this experiment, the conversion of

the heavy chain oligosaccharides from the high mannose precursor form to the complex type could be clearly resolved without digestion by endo H. For both antigens, the heavy chains exhibited a characteristic shift to a higher apparent molecular size upon processing of their oligosaccharides. A quantitative analysis of the kinetic data was obtained by densitometric scanning of the autoradiogram as shown in Fig. 2b. This figure clearly illustrates the profound difference in the rates of oligosaccharide processing for the two antigens. Half-time values were calculated to be 15 min for H-2K^k and 240 min for H-2D^k. Thus, the H-2D^k antigen is processed about 16 times more slowly than is the H-2K^k antigen.

Since the processing enzymes that confer endo H resistance have been localized in a number of tissues to the medial and trans regions of the Golgi apparatus (16, 17, 22-25) one possible explanation for the results is that there may be a 16-fold difference in the rates of intracellular transport of the two antigens at some point along the pathway from the RER to the cis Golgi region. If so, this difference should also be manifested in the rates of surface appearance of the antigens. Alternatively, the H-2D^k antigen may be relatively resistant to the action of processing enzymes but is transported to the cell surface at a rate similar to that of H-2K^k. In this case, one might expect to observe a population of H-2D^k molecules that remains sensitive to endo H digestion even after arrival at the cell surface. In order to address these possibilities further, the appearance of H-2K^k and H-2D^k at the cell surface was examined.

² For the purpose of this study, oligosaccharides that have been processed to the GlcNAc₁Man₃GlcNAc₂ stage and beyond (endo H-resistant forms) are considered to be of the complex type. No atypical high-mannose oligosaccharides that are resistant to endo H digestion have been detected for either H-2K^k or H-2D^k (21).

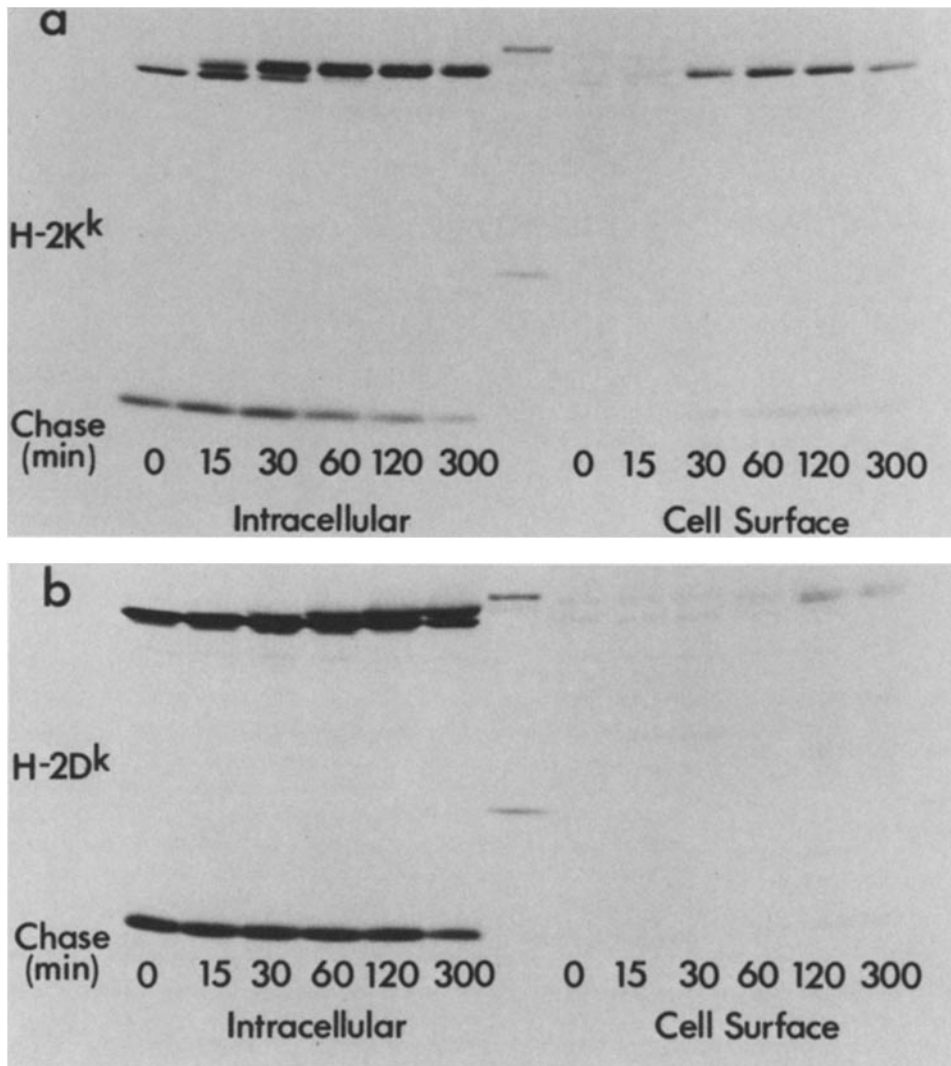


FIGURE 3 Appearance of H-2K^k and H-2D^k at the cell surface. Cells were pulsed for 10 min with [³⁵S]methionine and chased for various lengths of time. Radiolabeled class I antigens were recovered sequentially from the cell surface and intracellular locations using the two-step antibody protocol described in the text. The right half of each panel depicts antigen isolated from the cell surface at each chase time; the left half shows antigen isolated from the intracellular fraction at corresponding times. (a) Surface appearance of H-2K^k. (b) Surface appearance of H-2D^k. Murine IgG was included in the center lanes as a molecular weight standard (heavy chain, *M*_r 53,000; light chain, *M*_r 23,000).

Appearance of Newly Synthesized Class I Antigen at the Cell Surface

To detect the surface appearance of newly synthesized H-2K^k and H-2D^k, a two-step antibody protocol was used (see Materials and Methods). This method relies on the ability of antibodies to bind selectively to cell surface molecules upon incubation with intact cells. Briefly, aliquots of radiolabeled cells were removed after various periods of chase and incubated with antibody specific for either of the two antigens. Unbound antibody was washed out, and the cells were lysed in the presence of an excess of unlabeled cell extract. This reduces the likelihood of any residual free antibody binding to radiolabeled intracellular molecules exposed during lysis. Immune complexes were isolated with protein A-Sepharose (cell surface fraction), and then the lysates were subjected to a second round of antibody treatment to recover antigens unavailable to the first antibody (intracellular fraction).

Using this procedure, the surface appearance of H-2K^k was monitored as shown in Fig. 3*a*. It is clear that this antigen is first detectable on the cell surface after about 30 min of chase. In addition, the [³⁵S]methionine-labeled surface molecules exhibit the characteristic slower mobility expected for mature endo H-resistant species (compare the 30-min time points for the intracellular and cell surface fractions). Close inspection

of the cell surface fraction after short periods of chase (0 and 15-min time points) also reveals trace amounts of endo H-sensitive antigen. Rather than actually being present at the cell surface, this species most likely arises from the binding of intracellular antigen molecules to residual immunoglobulin that remains after the first antibody treatment. This apparently occurs even though lysis was conducted in the presence of an excess of unlabeled cell extract. Consequently, to confirm that the H-2K^k antigen is exclusively in the endo H-resistant form at the cell surface, unlabeled AKTB-1b cells were iodinated with ¹²⁵I and the antigen was isolated and digested with endo H (Fig. 4; lanes 1 and 2). Under conditions where the internal ribonuclease B standard is completely digested by the enzyme (not shown), all surface radioiodinated H-2K^k molecules are resistant to endo H treatment.

Another prominent feature of Fig. 3*a* is the presence of substantial amounts of endo H-resistant H-2K^k in the intracellular fraction. This could arise either from a large intracellular pool of mature or nearly mature antigen or by incomplete recovery of cell surface antigen by the first antibody treatment. In the latter case, any remaining cell surface antigen would be bound in the second antibody treatment and pooled together with the intracellular material. It is essential to distinguish between these possibilities because the occurrence of a large pool of endo H-resistant antigens within the

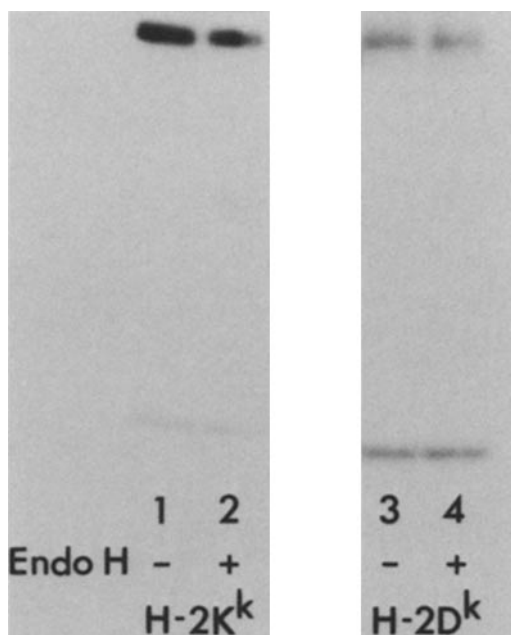


FIGURE 4 Digestion of cell surface antigens with endo H. AKTB-1b cells were radiolabeled vectorially with lactoperoxidase and Na-¹²⁵I. The cells were lysed, and class I antigens were isolated and subjected to digestion with endo H. Lanes 1 and 2, incubation of ¹²⁵I-H-2K^k in the absence or presence of endo H respectively. Lanes 3 and 4, incubation of ¹²⁵I-H-2D^k in the absence or presence of endo H, respectively. Ribonuclease B was added to all incubations as an internal control to ensure that the endo H was active. It was detected by staining the gel with Coomassie Blue before autoradiography.

cell would suggest a slow step in its intracellular transport at a point after most of the processing enzymes have acted, i.e., between the Golgi apparatus and the plasma membrane. Several different approaches were taken to deal with this question. The first approach involved the incubation of intact cells with proteases to remove only cell surface antigen, thereby permitting an examination of the remaining intracellular material. Conditions for digestion were sought by incubating radioiodinated cells with either trypsin, papain, or pronase for various lengths of time and at different temperatures followed by isolation of ¹²⁵I-H-2K^k and H-2D^k. Unfortunately, these experiments revealed that conditions required for digestion of the radiolabeled cell surface antigens also resulted in a dramatic decrease in cell viability (measured by dye exclusion and reduction in cell number). Apparently, these glycoproteins are oriented in the plasma membrane of AKTB-1b cells in a manner that renders them particularly resistant to proteolytic attack. Impermeable heterobifunctional cross-linking agents were also tested for their ability to react selectively with surface antigen. These negatively charged agents rapidly penetrated the cells and cross-linked both intracellular and cell surface molecules. However, two independent lines of evidence point to the conclusion that the endo H-resistant H-2K^k antigen in the intracellular fraction is primarily of cell surface origin. First, radioiodination of cells to label only surface proteins followed by the two-step antibody protocol succeeded in the recovery of only 25% of this antigen in the first step (data not shown). This is precisely the value obtained upon comparison of the amounts (by densitometry) of endo H-resistant H-2K^k present in the cell

surface and intracellular fractions in Fig. 3a (30–300-min time points). Assuming that the radioiodinated and metabolically labeled antigens have similar orientations at the plasma membrane, these results suggest that as early as 60 min of chase, essentially all of the newly synthesized H-2K^k has arrived at the cell surface. Further, subcellular fractionation experiments that are presented in the following section reveal that after 2.5 h of chase, all of the radiolabeled H-2K^k antigen is localized to the plasma membrane. Therefore, there does not appear to be a substantial intracellular pool of antigen that is resistant to endo H digestion.

The kinetics of surface appearance for the H-2D^k antigen are presented in Fig. 3b. It is apparent that the newly synthesized antigen first arrives at the cell surface after about 60 min of chase. As observed for H-2K^k, it migrates in the retarded fashion characteristic of mature, endo H-resistant molecules. This was confirmed by endo H digestion of H-2D^k isolated from radioiodinated AKTB-1b cells. All of the labeled cell surface antigen was resistant to the action of the enzyme (Fig. 4; lanes 3 and 4). This finding argues against the possibility mentioned previously, that the slowly processed H-2D^k antigen might be relatively resistant to the action of processing enzymes (perhaps for steric reasons) but is transferred rapidly to the cell surface. In this situation, one would have expected to detect a population of endo H-sensitive molecules at the surface of these cells.

The presence of large amounts of endo H-resistant antigen in the intracellular fraction was also observed for H-2D^k (Fig. 3b). Using the same criteria outlined above for the H-2K^k antigen, it became clear that this arises from the inability of the first antibody to bind to all of the cell surface molecules, even though the antibody is present in vast excess. When the two-step antibody binding protocol was applied to radioiodinated cells, only 18.8% of the surface antigen could be recovered in the first step (data not shown). This compares quite closely to the percentage of endo H-resistant antigen present in the cell surface fractions of pulse-chased cells (12%; taken from the 300-min point in Fig. 3b). In addition, subsequent subcellular fractionation experiments (see below) revealed that after 2.5 h of chase all of the endo H-resistant H-2D^k antigen is present in the plasma membrane fractions. Thus there does not appear to be a substantial pool of endo H-resistant antigen within the cells.³

The presence of contaminating cell surface antigens in the intracellular fraction prevents a precise determination of the rates for appearance of newly synthesized H-2K^k and H-2D^k at the cell surface. However, the data still permit some important conclusions to be drawn. First, it is clear that the two antigens are transferred to the surface of the cell at markedly different rates. Although both first arrive after 30–60 min of chase, the H-2K^k antigen arrives as a discrete population; all of the labeled molecules are present at the plasma membrane after 2.5 h of chase and probably as early as 60 min. The H-2D^k antigen, however, requires many hours to accumulate on the cell surface. Even after 5 h of chase, roughly half of the labeled molecules are still present within the cell in an endo H-sensitive form. Second, the kinetics of surface appearance

³ It should be noted, however, that endo H-resistant forms of both H-2K^k and H-2D^k can be detected transiently within the cells. As shown in Fig. 3a (15-min chase) for H-2K^k and in Fig. 3b (30-min chase) for H-2D^k, the endo H-resistant form can be observed in the intracellular fraction with none present at the cell surface.

of these two antigens resemble the kinetics whereby they acquire endo H-resistance, i.e., the H-2K^k antigen is rapidly and quantitatively processed, whereas the H-2D^k antigen is processed slowly over many hours (Fig. 2*b*). In other words, once endo H-resistant antigen is formed it does not accumulate substantially within the cell but is transported quickly to the surface. This suggests that the rate-limiting step in the slow transfer of H-2D^k to the cell surface occurs before the formation of endo H-resistant oligosaccharides, i.e., somewhere along the pathway from the RER to the cis Golgi region.

Subcellular Location of H-2D^k Accumulation

To define more precisely the rate-limiting step in the intracellular transport of H-2D^k, subcellular fractionation experiments were conducted to identify the site where it accumulates. Cells pulsed for 10 min with [³⁵S]methionine or pulsed and then chased for 2.5 h were homogenized, and a postnuclear membrane fraction was prepared. This was applied to a sucrose density gradient and centrifuged to equilibrium. Fig. 5*a* summarizes the activities across the gradient of several enzymes that are accepted to be markers of particular subcellular organelles. As expected, the ER marker, glucosidase I, distributes at higher densities than does 5'-nucleotidase, the marker for plasma membrane (26, 27). Although the two activities overlap, their distributions are reproducible and quite distinctive. GlcNAc-transferase I and sialyltransferase activities were also assayed as markers of the medial and trans Golgi regions. In this cell line, both activities are distributed across the gradient in an identical fashion with the highest activity at fraction 19 and substantial activity at higher densities. This unusual profile, which is clearly distinct from that of the ER marker, probably arises from aggregation of the Golgi membranes. However, it could not be altered by raising the salt concentration of the buffers or by changing homogenization conditions. Neither the H-2K^k nor the H-2D^k antigen exhibited this distinctive distribution, and because these markers tended to obscure the other enzyme profiles, they were excluded from the figure.

Fig. 5*b* shows the distributions across the gradient of radiolabeled H-2K^k and H-2D^k after a 10-min pulse. The two antigens exhibit very similar profiles that are almost identical to that of the ER marker, glucosidase I. This result is consistent with the fact that cell surface glycoproteins are inserted into the RER membrane during translation (2, 28). As expected, both antigens are completely sensitive to digestion by endo H across the entire gradient (not shown). However, after a 2.5-h chase, the H-2K^k antigen has a markedly different distribution. It displays the distinctive peaks observed for the plasma membrane marker, thereby confirming its cell surface location (Fig. 5*c*). Consistent with previous experiments, this antigen is now completely endo H-resistant (not shown). In sharp contrast, the slowly transported H-2D^k antigen exhibits a bimodal distribution after the chase (Fig. 5*d*). The mature, endo H-resistant molecules distribute with the plasma membrane, whereas the endo H-sensitive molecules are found associated with the ER. We cannot completely exclude the possibility that a portion of endo H-sensitive H-2D^k molecules are present in Golgi membranes. However, the amount of antigen detected in fractions 19 and higher indicate that this would be a small percentage of the total. The fact that the intracellular form of H-2D^k accumulates in the ER dem-

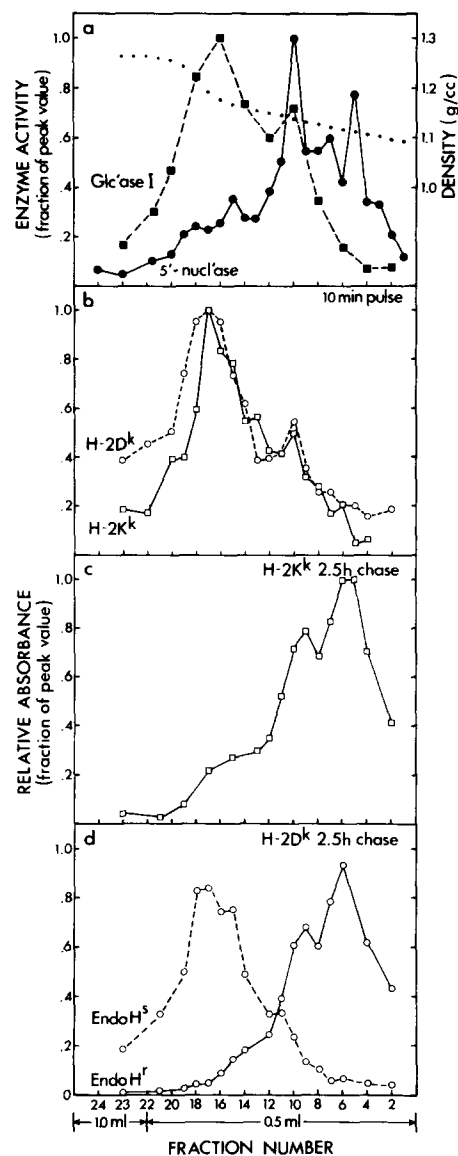


FIGURE 5 Subcellular localization of pulse-chase labeled H-2K^k and H-2D^k antigens. AKTB-1b cells were either pulsed for 10 min with [³⁵S]methionine or pulsed and then chased with unlabeled methionine for 2.5 h. As described in Materials and Methods, postnuclear supernatant fractions were prepared and applied to discontinuous sucrose gradients. After centrifugation to equilibrium, radiolabeled antigens were isolated from the gradient fractions and analyzed by SDS PAGE. The autoradiograms were scanned to quantitate the amounts of endo H-sensitive and resistant antigens in the various fractions. A sample of nonradioactive cells was also subjected to the fractionation procedure to assay the activities of the marker enzymes, α -glucosidase I (26) and 5'-nucleotidase (27). (a) Distribution across the gradient of α -glucosidase I activity ([■]; total activity in peak fraction is 0.02 U as defined in reference 26) and 5'-nucleotidase activity ([●]; total activity in peak fraction is 48.5 nmol AMP hydrolyzed/h). Recoveries of enzymatic activity were 72% for α -glucosidase I and 87% for 5'-nucleotidase relative to the starting membranes. Sucrose densities were determined from refractive indices and are shown at the top of the panel (---). (b) Distributions of H-2K^k and H-2D^k after a 10-min pulse. (c) Distribution of H-2K^k after a 2.5-h chase. (d) Distribution of H-2D^k after a 2.5-h chase.

onstrates that the rate-limiting step in its transport to the cell surface is its exit from this organelle.

One possible explanation for the slow transport of H-2D^k

TABLE I. Ratio of Heavy Chain to β_2 -Microglobulin for the H-2D^k Antigen Isolated from Plasma Membrane- and Endoplasmic Reticulum-enriched Fractions of AKTB-1b Cells

	Fraction number	Heavy chain/ β_2 -microglobulin
Plasma membrane-enriched fractions	4	6.5
	6	5.8
	8	4.8
	10	5.5
	12	4.2
Endoplasmic reticulum-enriched fractions	14	5.8
	16	6.3
	18	5.4

The autoradiograms used to determine the subcellular distribution of the H-2D^k antigen in pulse-chase labeled AKTB-1b cells (Fig. 5 d; see legend for details) were scanned densitometrically to examine the ratio of heavy chain to β_2 -microglobulin in the various fractions.

could be that those molecules that are retained in the ER are not associated with β_2 -microglobulin. Binding of β_2 -microglobulin to heavy chains is thought to be a prerequisite for transport to the cell surface because in Daudi cells, a line in which the gene for β_2 -microglobulin is not expressed, no class I antigens are present at the surface (29). To examine this possibility, the H-2D^k antigen isolated from the plasma membrane and ER regions of the sucrose gradient (Fig. 5 d) was analyzed to determine the ratio of heavy chain to β_2 -microglobulin. The results of this analysis are shown in Table I. No increase in the ratio could be detected in those fractions enriched for ER relative to those enriched for plasma membrane. Therefore, it is unlikely that the slow intracellular transport of H-2D^k can be attributed to a decreased binding of β_2 -microglobulin.

DISCUSSION

In this study, we demonstrate how limited differences in the structure of plasma membrane glycoproteins can exert a profound influence on the rates at which they are transported to the cell surface. The H-2K^k antigen produced by AKTB-1b cells is transported rapidly such that all molecules reach the plasma membrane as early as 1 h after synthesis. However, the H-2D^k antigen, which shares ~80% sequence homology with H-2K^k, appears at the cell surface very slowly. Roughly half of the molecules remain intracellular 5 h after synthesis. The determination of these rates was complicated by the fact that the antibodies used to detect the antigens at the cell surface could not recover them quantitatively. Neither monoclonal antibodies nor alloantisera were effective in this regard. Perhaps a portion of the antigens is sterically inaccessible to the antibodies or, alternatively, the antibodies do not bind with sufficient avidity, resulting in dissociation of the complexes before their isolation. Support for the former view is provided by the fact that the cell surface antigens were not susceptible to the action of a number of different proteases under conditions that retained cell viability. Consequently, a determination of cell surface antigen recovery was required before estimates of the rates of surface appearance could be made.

Two lines of evidence indicate that the rate-limiting step in the transport of H-2D^k to the plasma membrane occurs at the level of the ER. First, the rate of surface appearance for this

antigen closely resembles the rate at which its covalently associated oligosaccharides acquire resistance to digestion by endo H (half-time of 4 h). Since N-linked oligosaccharides are processed to endo H-resistant forms in the medial and trans cisternae of the Golgi apparatus, this result suggests that the slow step in H-2D^k transport occurs before this point, i.e., at some point along the pathway between the RER and the cis Golgi region. Second, subcellular fractionation experiments conducted on pulse-chased cells clearly identified the ER as the site where the endo H-sensitive form of H-2D^k accumulates. These experiments also demonstrated that mature, endo H-resistant antigen could not be detected within the cell; essentially all of it was present at the cell surface. Therefore, there does not appear to be an additional slow step in transport occurring at a late stage in maturation of the antigen, i.e., between the Golgi apparatus and the plasma membrane. For the rapidly transported H-2K^k antigen, subcellular fractionation revealed that after the same length of chase (2.5 h), no antigen could be detected within the cell. All of it was present in endo H-resistant form in the plasma membrane fractions.

These results are consistent with our previous studies on the nature of the oligosaccharide chains of the H-2K^k and H-2D^k antigens synthesized by AKTB-1b cells (21). Sialylated biantennary oligosaccharides were the predominant species detected at the two glycosylation sites of mature H-2K^k and at the three sites of mature H-2D^k. However, when the antigens were labeled with relatively short pulses of [³H]mannose (5 h), 70–80% of the radioactivity incorporated into total cellular H-2D^k was present in molecules possessing a single type of high-mannose oligosaccharide compared to 10–18% for H-2K^k. Analysis of the isolated oligosaccharide indicated that the probable composition was Man₆GlcNAc₂. The large proportion of radiolabeled H-2D^k carrying this precursor oligosaccharide reflects the accumulation of this antigen in the ER. A variety of studies have demonstrated that glycoproteins localized to the ER exhibit oligosaccharide-processing intermediates ranging in size from Man₆GlcNAc₂ to Man₉GlcNAc₂ (3, 30–32).

The lack of a significant accumulation of H-2K^k within AKTB-1b cells contrasts sharply with the findings of Le and Doyle (33) who estimated that 80% of both H-2K^k and H-2D^k antigens exist as an intracellular pool in the splenocytes of AKR mice. This value was obtained by neuraminidase treatment of intact, [³⁵S]methionine-labeled cells and quantitation of the fraction of antigen molecules not digested by the enzyme. The basis for the discrepancy is not clear. Perhaps in their study not all of the radiolabeled cell surface antigen was accessible to the action of neuraminidase and consequently appeared to have an intracellular location. Alternatively, there is the intriguing possibility that the transport machinery of splenocytes and lymphoma cells may differ in their interactions with H-2K^k resulting in altered rates of intracellular transport in the two types of cells. Clearly, further work will be required to resolve this question.

The basis for the differential retention of the closely related H-2K^k and H-2D^k antigens in the ER of AKTB-1b cells is not known. We examined the possibility that the accumulation of H-2D^k in this organelle could be due to a decreased binding to β_2 -microglobulin. However, no difference could be detected in the ratio of heavy chain to β_2 -microglobulin for antigen isolated from either the ER or the plasma membrane. Further, the H-2D^k remaining in the ER over the time period studied

does not appear to be a subpopulation of newly synthesized antigen that is insoluble or otherwise structurally distinct from those molecules that reach the cell surface. Extended chase experiments of 22 h duration revealed that at least 85% of the radiolabeled H-2D^k could be converted to an endo H-resistant form (data not shown).

Our results can best be explained by a receptor-mediated mechanism in which one or more receptors localized to the ER membrane mediates the selective transport of newly synthesized proteins out of this organelle to the Golgi apparatus. Such a mechanism has been proposed previously by Lodish and co-workers (3) to explain differential transport rates of several hepatic secretory proteins and by Fitting and Kabat (4) to account for differences in the transfer of two viral membrane glycoproteins to the plasma membrane. In both studies, slowly transported glycoproteins were retained in the ER. According to the model, rapidly transported proteins such as H-2K^k would bind to their receptor(s) with high affinity and the resulting complexes would be incorporated into transport vesicles that bud off from specialized regions of the ER for rapid transit to and subsequent fusion with the Golgi apparatus. Slowly transported proteins such as H-2D^k would bind weakly, thereby leading to an accumulation in the ER.

The concept of specific transport receptors is attractive because it accounts for the remarkable selectivity in the transport of closely related molecules observed in our study. Such selectivity is clearly incompatible with alternative mechanisms such as transport via passive membrane flow between organelles (1). Also, a receptor-mediated process is likely to provide an initial sorting of transported molecules from resident components of the ER membrane that presumably do not bind to the receptor. This is clearly an essential requirement during the export of proteins to various cellular destinations, otherwise the compositional integrity of organelles such as the Golgi apparatus, lysosomes, and plasma membrane would be lost (22). Through a process akin to receptor-mediated endocytosis, the clustering of receptor-protein complexes in specialized regions of the ER membrane could exclude resident components of the membrane and thus effect a substantial purification. However, despite the increasing body of indirect evidence favoring a transport receptor mechanism for the exit of secretory and plasma membrane proteins from the ER, there has not as yet been any direct demonstration that such receptors exist. Clearly, future efforts must be directed toward their identification and characterization.

An important aspect of this study is that the two membrane glycoproteins shown to possess widely different transport kinetics are closely related in structure. Although their amino acid sequences are not yet known in their entirety, by analogy to other class I antigens that have been completely sequenced, the heavy chains of the H-2K^k and H-2D^k antigens share at least 80% homology (8). Direct evidence supporting this conclusion is provided by the fact that only a single amino acid difference has been detected between the two antigens within the first 30 residues from their amino termini (34, 35). Presumably, the determinants that dictate the different rates at which these antigens exit the ER reside in nonhomologous regions of their polypeptide chains.

Whether such determinants are formed solely by the polypeptide backbone of these antigens is not clear. A number of studies using inhibitors of glycosylation or of oligosaccharide processing have indicated that the covalently associated oli-

gosaccharides of glycoproteins may also play a role in the transport process (36–38). It is unlikely that the different rates at which H-2K^k and H-2D^k exit the ER can be attributed to differences in the structures of their oligosaccharide chains. Our previous investigations have shown that after a 5-h pulse with [³H]mannose, the precursor forms of both antigens carry similar oligosaccharides having the composition Man₆GlcNAc₂ (21). However, the H-2K^k and H-2D^k antigens do differ in the number of glycosylation sites that they possess. Both antigens are glycosylated at residues 86 and 176, but H-2D^k has one additional site (21). It is possible that the additional oligosaccharide chain in the H-2D^k molecule influences the rate of transport of this antigen through the cell. Clearly, any future assessments of the relative roles played by the polypeptide and carbohydrate moieties of glycoproteins in the transport process will require the availability of molecules with defined alterations in each component.

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