N-Linked Oligosaccharides of the H-2D^k Histocompatibility Protein Heavy Chain Influence Its Transport and Cellular Distribution†

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ABSTRACT: The H-2K and H-2D proteins encoded by the K and D region of the major histocompatibility complex of the mouse were isolated by immunoprecipitation with specific antisera and resolved by two-dimensional gel electrophoresis. Of these two polypeptides, the H-2D^k glycoproteins isolated from macrophages of C3H/HeHa mice exhibit distinct cell surface and cytoplasmic forms although they share a strong degree of homology in the polypeptide backbone. Structurally they differ in their oligosaccharide structures. The structure of the oligosaccharides on the intracellular forms is of the high mannose type while the same structures on the cell surface forms are of the complex type. In the absence of all three oligosaccharide side chains, the unglycosylated polypeptides are expressed on the cell surface. In contrast, polypeptides containing one, two, or all three oligosaccharide side chains of the high mannose type are not transported to the cell surface. Cell surface expression of these glycoproteins requires processing of the oligosaccharide side chains from the high mannose form to the complex type. However, not all oligosaccharide antennae have to be terminally modified since H-2D^k glycoproteins synthesized in the presence of oligosaccharide-processing enzyme inhibitors such as swainsonine or monensin are also transported to the cell surface. H-2D^k glycoproteins containing oligosaccharide structures of the complex type but lacking terminal sialic acids are found on the cell surface, suggesting that sialylation is not required for transport. These results indicate that the oligosaccharide structures of the H-2D^k glycoproteins act to influence their cellular distribution.

We have previously shown that the H-2D^k glycoproteins encoded by the D region of the major histocompatibility complex (Klein, 1979) of the mouse isolated from spleen cells of C3H/HeHa mouse exhibit distinct intracellular and cell surface forms (Le & Doyle, 1982). Only the larger and more acidic subset of the H-2D^k is susceptible to neuraminidase digestion, indicating that the smaller and more basic form is not sialylated. Moreover, the nonsialylated forms which comprise half of the total H-2D^k are not accessible to cell surface iodination, suggesting that they may be part of some intracellular membrane compartment. This population of H-2D^k may be in transit either in the Golgi or in the rough endoplasmic reticulum awaiting processing into its final mature forms; in lymphocytes, this, however, does not appear to be the case. From pulse–chase experiments utilizing [^{35}S]-methionine as label, not all the nonsialylated forms of the H-2D^k are converted into the mature fully sialylated forms even after an 18-h chase. Limited protease digestion of the two subsets of the H-2D^k showed that both subsets share a strong degree of homology in the polypeptide backbone. Both forms of the H-2D^k must initially follow identical routes from their site of synthesis in the rough endoplasmic reticulum and then diverge on their way to the cell surface. Approximately half of the H-2D^k molecules will remain inside while the other half travels to the cell surface. For proper intracellular sorting and transport of the H-2D^k, specific structural characteristics within the molecule must exist to mediate the delivery of these glycoproteins to their distinct cellular location.

In this paper we will show that in macrophages the H-2D^k proteins also exist as distinct cell surface and cytoplasmic forms. Only the H-2D^k polypeptides containing oligosaccharide structures of the complex type are expressed on the cell surface. In contrast those polypeptides possessing oligosaccharide structures of the high mannose type remain in some yet unknown intracellular pool. While sialylation is not required for cell surface expression, processing of this oligosaccharide structure from a high mannose structure to an endoglycosidase H resistant form is required for cell surface expression. These results suggest that following the transfer of the high mannose oligosaccharide structure to the growing H-2D^k polypeptide chain processing of this structure to an endoglycosidase-resistant form may be a prerequisite and limiting step in triggering the transport of the H-2D^k glycoproteins via the Golgi to the cell surface. Determinants inherent in the H-2D^k glycoproteins that may influence the cellular distribution of these glycoproteins could reside at least in part on the oligosaccharide moieties.

Materials and Methods

Mice. C3H/HeHa were from the West Seneca Laboratories, Buffalo, NY.

Antisera. The antisera were obtained from the Immunology and Immunochemistry Branch of the National Institutes of Health: D-23, anti-K^k, and D-32, anti-D^k.

Preparation and Labeling of Cells. Macrophages were prepared and radiolabeled essentially as described by Le & Doyle (1982). Four days after the intraperitoneal injection of 1 mL of thioglycolate broth (Difco), peritoneal macrophages were collected and seeded at 10^7 cells/25-cm^2 flask in Dulbecco's modified minimal essential medium containing 10% heat-inactivated fetal calf serum (Gibco), buffered with 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes),[1] and supplemented with L-glutamine (4 mM), penicillin, and streptomycin (DMEM/FCS). After a 2-h incu-

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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bation at 37 °C in a 10% CO_2 incubator and again 15 h later, the culture flasks were washed extensively with the same medium to remove nonadherent cells. Such preparation yields a very homogeneous population of adherent macrophages with viability greater than 98%. For radiolabeling, unless indicated otherwise, [^{35}S]methionine (1000 Ci/mmol) was added to a final specific activity of 150 μCi/mL in DMEM/FCS containing only 10% of the original concentration of methionine (low methionine) required for growth and incubated at 37 °C in a 10% CO_2 incubator for 15 h. Incorporation of labels was stopped by removal of medium, and the cells were washed 3 times with Earle's balanced salt solution. For the pulse and chase experiment, [^{35}S]methionine was added to a final specific activity of 2.5 mCi/mL low methionine culture medium without fetal calf serum. At the end of the 10-min pulse, the cells were washed twice with DMEM/FCS and then maintained in the same DMEM/FCS throughout the entire chase period. Cell viability at the end of the labeling or chase period was greater than 95%.

Inhibition of Glycosylation. Flasks (25 cm^2) containing about 10^7 macrophages were incubated with tunicamycin (2 μg/mL), swainsonine (10 mM), or monensin (1 μM) in DMEM/FCS for 2 h and then labeled with [^{35}S]methionine in the presence of the inhibitor for another 18 h. At the end of the labeling period, the cells were washed twice with Earle's balanced salt solution and then processed for Immunoprecipitation. The concentrations of the inhibitors used were chosen to yield maximum inhibition of glycosylation while cell viability remained greater than 90%.

Endoglycosidase H Digestion. Radioactive spots corresponding to the H-2 proteins on a two-dimensional sodium dodecyl sulfate– (SDS–) polyacrylamide electrophoresis gel were cut out, loaded into the wells of a one-dimensional SDS–polyacrylamide gel electrophoresis, and equilibrated in 0.125 M Tris-HCl, pH 6.5, containing 0.1% SDS and 10% glycerol with several change of the same buffer over 30 min. The equilibrated gel pieces were then overlayed with endoglycosidase H at 10 milliunits per well. The protein content of the gel pieces was then electrophoresed into the stacking gel. The current was turned down to zero and the temperature of the gel raised to 37 °C when the dye front reached the interphase between resolving and stacking gel. Endoglycosidase H digestion was allowed to proceed for 30 min at 37 °C. The digested proteins were then electrophoresed into the 10% SDS–polyacrylamide resolving gel. This procedure was adapted from the method originally described by Cleveland et al. (1977) for peptide mapping of proteins by limited protease digestion.

Neuraminidase Digestion. Unless indicated otherwise intact [^{35}S]methionine-labeled macrophages were removed from the incubator, washed twice with DMEM, pH 6.5, and then exposed to the same medium containing 25 milliunits/mL of neuraminidase for 10 min at 37 °C. At the end of the enzymatic treatment, the cells were again washed twice with ice-cold DMEM/FCS (pH 8.0) and then processed for immunoprecipitation. To determine the timing of the appearance of newly synthesized H-2 glycoproteins on the cell surface, macrophages were initially pulsed with [^{35}S]methionine (250 μCi/mL) in serum-free medium and low methionine for 20 min and then chased with DMEM/FCS for up to 60 min. At specific time points during the chase period, intact macrophases were removed from the incubator, washed, exposed to neuraminidase, and processed as described above. In each case cell viability always remained greater than 90%.

Immunoprecipitation of Radiolabeled Cell Proteins and Two-Dimensional Gel Electrophoresis. All of the procedures described by Le & Doyle (1982) were followed.

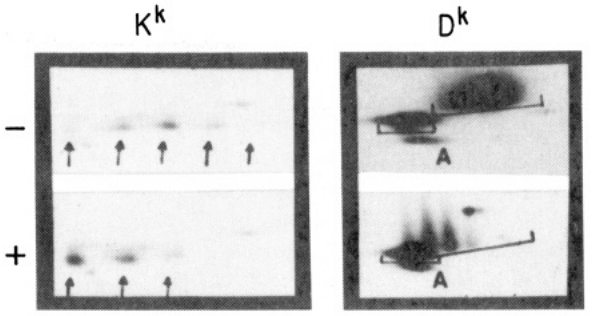

FIGURE 1: Neuraminidase digestion of cell surface associated H-2K^k and H-2D^k. Shown are proteins immunoprecipitated from neuraminidase-treated intact macrophages that were labeled continuously with [^{35}S]methionine for 15 h. In this and all subsequent figures, A denotes actin, arrows denote H-2K^k, and brackets denote H-2D^k. Also, only the relevant portion of the two-dimensional gel is shown. (–) No treatment; (+) plus neuraminidase. The basic end of the gel is toward the left.

Reagents. Endoglycosidase H was purchased from the Division of Laboratories and Research, Albany NY. Tunicamycin, neuraminidase, and monensin were from Calbiochem-Behring. Swainsonine was a generous gift from Dr. H. Segal, Department of Biological Sciences, SUNY/Buffalo.

RESULTS

Identification of Intracellular and Cell Surface Forms of the H-2D Antigens. H-2D^k antigens isolated from macrophages of C3H/HeHa mice were shown to be heterogeneous both in size and charge; the larger and more acidic forms are derived from the smaller and more basic forms by addition of sialic acid residues (Le & Doyle, 1982). Unlike their counterparts in lymphocytes, all the larger and more acidic forms of H-2D^k represent cell surface forms because digestion of intact [^{35}S]methionine-labeled macrophages with neuraminidase cause a complete shift of all the larger and acidic forms toward the more basic end of the two-dimensional gel (Figure 1). Desialylated H-2D^k glycoproteins migrate as a family of three or more clearly visible spots whose electrophoretic mobility is very different compared to the fully sialylated H-2D^k. Comigrating with the two more basic spots of this family of desialylated H-2D^k proteins is the spot corresponding to the smaller and more basic form of the H-2D^k proteins. The electrophoretic mobility of this smaller and more basic spot is not affected by neuraminidase as determined by its relative position to the contaminant actin (denoted by A). Similar results were obtained when the H-2D^k glycoproteins were first immunoprecipitated and then exposed to neuraminidase. These results suggest that the larger and more acidic form of the H-2D^k is derived from the smaller and more basic form. We will show later that the smaller and more basic form of H-2D^k is also not accessible to cell surface iodination (see Figures 7 and 9). This smaller and more basic form, most probably like its counterpart in lymphocytes, represents the cytoplasmic form of H-2D^k. In a similar fashion, desialylated H-2K^k proteins also differ from the fully sialylated H-2K^k proteins by virtue of their electrophoretic mobility as evidence by the disappearance of the two more acidic spots concomitant with the appearance of one additional very basic spot. As a control the neuraminidase treatment was carried out at 4 °C instead of 37 °C to ensure that only cell surface associated H-2 molecules are digested by the enzyme. The results thus obtained were the same.

To show that the two forms of the H-2D^k are related to one another, the kinetics of labeling of these two forms were de-

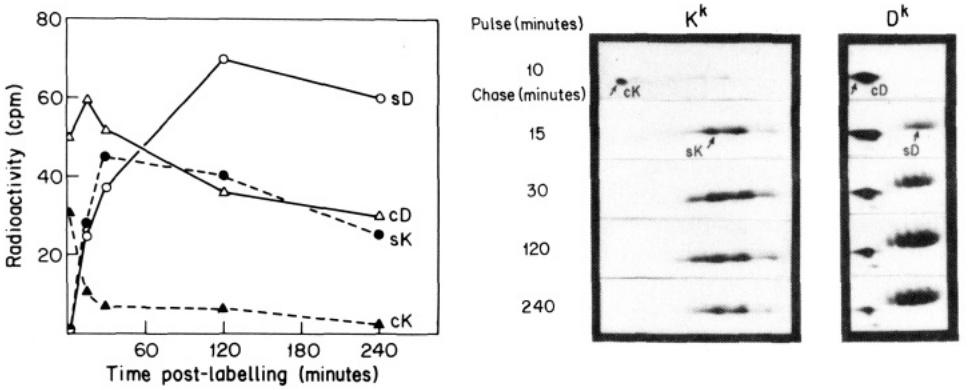

FIGURE 2: Timing of the conversion of the cytoplasmic form to the cell surface form of H-2D and H-2K antigens. Isolated macrophages were labeled with [^{35}S]methionine for 10 min. At the end of the labeling period, the cells were washed twice with DMEM containing fetal calf serum and then chased in the presence of unlabeled methionine for up to 4 h. At indicated time points, the H-2 proteins were immunoprecipitated and resolved by two-dimensional gel electrophoresis. Spots corresponding to the cytoplasmic and cell surface forms were cut out from the gels and the amount of radioactivity incorporated into these spots determined by liquid scintillation spectrometry. sK and sD denote surface H-2K and H-2D, respectively. For this and subsequent figures, the cytoplasmic form of H-2D is cD. cK is used to indicate the basic form of H-2K seen at the end of the 10-min pulse with [^{35}S]methionine.

termined in a pulse–chase experiment. At the end of a 10-min pulse with [^{35}S]methionine most of the label is confined to the smaller and more basic form of the H-2D^k glycoproteins. However, not all of the [^{35}S]methionine-labeled H-2D^k molecules present after a 10-min pulse can be chased into the larger and more acidic forms. In contrast, all H-2K^k present at the end of the short pulse can be converted to the mature form. At the end of a 4 h chase period, at least half of the nonsialylated form of H-2D^k is still present in the macrophages (Figure 2). In addition, there is no significant increase in the amount of radioactivity recovered in the larger and acidic form beyond the 2-h chase period. Since all [^{35}S]methionine was removed from the cultures after the 10-min pulse, the [^{35}S]-methionine incorporated into the larger and more acidic form must be derived from the smaller and more basic form, indicating that conversion of the more basic form to the more acidic form did take place. This is consistent with our earlier report in which peptide mapping by limited protease digestion showed that the two forms of H-2D^k share a strong degree of homology in the polypeptide backbone (Le & Doyle, 1982).

To determine the time required for newly synthesized H-2 antigens to reach the cell surface, macrophages were first labeled with [^{35}S]methionine for 20 min and then chased for up to 1 h in the absence of the radioactive label. Intact macrophages at specific time points during the chase period were removed and exposed to neuraminidase. Changes in electrophoretic mobility of the H-2 proteins at a specific time point as a result of the enzymatic digestion is indicative of their presence on the cell surface. Between 30 and 45 min is required for the newly synthesized H-2 glycoproteins to reach the cell surface as revealed by the accessibility of newly synthesized H-2 glycoproteins to cell surface neuraminidase digestion (Figure 3). This is consistent with our observations that conversion of newly synthesized H-2 glycoproteins to the mature form occurs as early as 15 min into the chase period or may be earlier (Figure 2) while newly synthesized H-2 appears at the cell surface within 45 min. These results, in addition to the observation that not all the smaller and more basic form of the H-2D^k can be chased into the larger and more acidic form, suggested to us that there are two forms of H-2D^k. The smaller and more basic spot represents the cytoplasmic form while the larger and more acidic spots are the cell surface forms. The inaccessibility of the smaller and more basic form of H-2D^k to cell surface iodination (refer to Figures 7 and 9) further supports this observation. In contrast, all [^{35}S]methionine-labeled H-2K^k proteins are accessible to

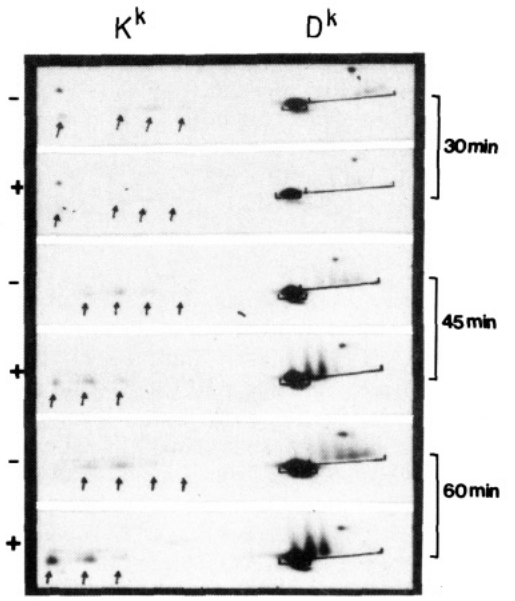

FIGURE 3: Timing of appearance at the cell surface of newly synthesized H-2 glycoproteins. Shown are the two-dimensional gel electrophoretic patterns of H-2 glycoproteins immunoprecipitated from [^{35}S]methionine-labeled, neuraminidase-treated intact macrophages at specific time points during the chase period. The time points indicated represent the length of time in which the 20-min pulse with [^{35}S]methionine, the chase period and the 10-min treatment with neuraminidase are included (i.e., $\pm_{60}$ = 20-min pulse + 30-min chase + 10-min neuraminidase). Procedures were as described under Materials and Methods. (+) refers to those cells treated with neuraminidase; (−) denotes control.

cell surface neuraminidase digestion (Figures 1 and 3), indicating that following their synthesis all H-2K^k proteins are transported to the cell surface. There is no significant intracellular pool of H-2K^k.

The two forms of H-2D^k differ in the structure of their carbohydrate side chains. Only the smaller and more basic form of H-2D is sensitive to endoglycosidase H digestion, indicating that its oligosaccharide structure is of the high mannose type (Figure 4). Since this is only a limited digestion with endoglycosidase H, the ladder of spots represent an incomplete digestion of the oligosaccharides side chain present on the H-2D^k polypeptides. Longer exposure to endoglycosidase changes this ladder of spots to a single spot, the molecular weight of which is approximately 35 000 (result not shown). Inhibition of glycosylation with tunicamycin also

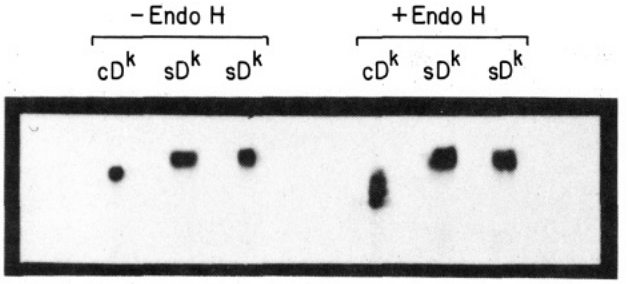

FIGURE 4: Sensitivity of the cell surface and cytoplasmic forms of H-2D^k to endoglycosidase H. Spots corresponding to the cell surface and cytoplasmic forms of H-2D^k immunoprecipitated from [^{35}S]-methionine-treated macrophages were cut from the two-dimensional gel and digested with endoglycosidase H as described under Materials and Methods. Endoglycosidase H (10 milliunits) was added as indicated.

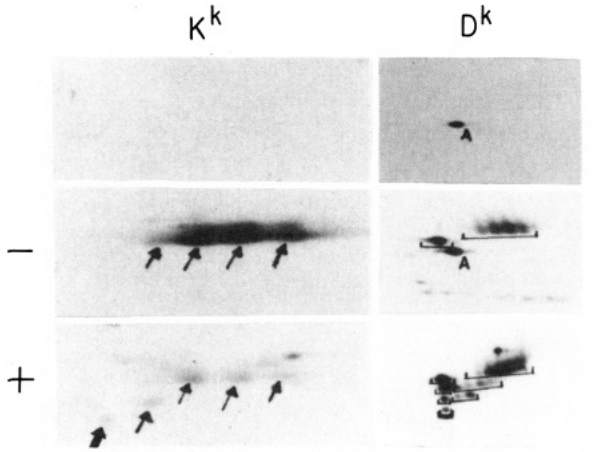

FIGURE 5: Inhibition of glycosylation by tunicamycin. Shown are proteins immunoprecipitated from [^{35}S]methionine-labeled macrophages of C3H/HeHa mice treated or not treated with tunicamycin. The first panel represents proteins immunoprecipitated from [^{35}S]-methionine-labeled C3H/HeHa macrophages with preimmune serum. In the third panel [labeled with (+)] the macrophages were pretreated with tunicamycin at 2 μg/mL prior to the addition of [^{35}S]methionine whereas in the second panel [labeled with (–)] the cells were not treated with tunicamycin. The antisera used in the second and third panel were D-32 (anti-H-2D^k) and D-23 (anti-H-2K^k). In each case, the cells were labeled with [^{35}S]methionine in the presence or absence of the inhibitor for 15 h. H-2K proteins are indicated by arrows pointing up; brackets denote H-2D proteins. The nonglycosylated precursors for H-2K and H-2D are indicated by heavy arrows and heavy brackets, respectively.

reveals a ladder of spots. Shown in Figure 5 are the H-2D^k glycoproteins immunoprecipitated from macrophages that were pretreated with tunicamycin at 2 μg/mL for 2 h and then labeled with [^{35}S]methionine in the presence of the inhibitor for 15 h. Under these conditions inhibition of glycosylation is incomplete. The smallest, single spot in the family of H-2D^k spots isolated from tunicamycin-treated macrophages has an apparent molecular weight of approximately 35 000. This molecular weight is consistent with the polypeptide size of the class 1 antigens as deduced from the DNA sequence coding for these molecules and from our endoglycosidase H digestion experiment. The smallest of this family of H-2D^k spots is believed to represent the unglycosylated polypeptide. As the nonglycosylated form of the H-2D^k polypeptide backbone acquires one or more *N*-glycans, the precursor polypeptides increases in molecular weight. This increase in molecular weight is approximately 3000 which is consistent with the reported molecular weight of one complete *N*-glycan after SDS–polyacrylamide gel electrophoresis. Once sialylated, the glycoproteins exhibit the multispot pattern typical of the

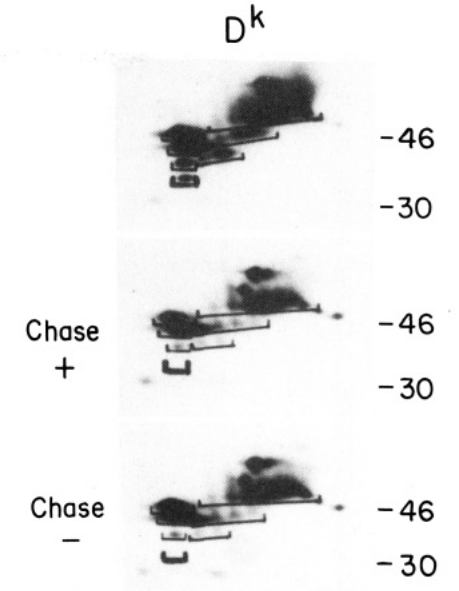

FIGURE 6: Fate of H-2D^k synthesized in the presence of tunicamycin following an 8-h chase in the presence or absence of the inhibitor. The first panel represents proteins immunoprecipitated from macrophages that were pretreated with tunicamycin (2 μg/mL) and then labeled with [^{35}S]methionine in the presence of the inhibitor for 15 h. The second panel consists of proteins immunoprecipitated from tunicamycin-treated [^{35}S]methionine-labeled macrophages following an 8-h chase with unlabeled methionine in the presence of tunicamycin. In the third panel the chase was conducted in the absence of tunicamycin. Brackets indicate H-2D.

mature form. We conclude on the basis of the result from the tunicamycin inhibition of glycosylation together with results from the pulse–chase experiment and the neuraminidase digestion and peptide mapping by limited proteases digestion experiments (Le & Doyle 1982) that the smaller, more basic form and the larger and more acidic form of H-2D^k are related and share similar polypeptide backbones. From these types of experiments producing partial inhibition of glycosylation we can also estimate that H-2D^k has at least 3 *N*-glycans. In contrast H-2K^k has two *N*-linked oligosaccharides.

Factors Involved in the Cellular Distribution of the H-2D Antigens. Both cytoplasmic and cell surface forms of the H-2D glycoproteins differ only in the structure of their oligosaccharide side chain. The high mannose form of the H-2D^k glycoproteins remains inside the cell whereas the endoglycosidase H resistant form, presumably possessing complex oligosaccharide structure, is transported to the cell surface. In the event the oligosaccharide structure contains information important in intracellular traffic control and direction, alteration of its structure in vivo could affect the delivery of these molecules to their correct destination. By removing the N-linked oligosaccharide structure that we believe contains the recognition marker necessary for intracellular sorting, we could effectively eliminate the intracellular form. Moreover, by use of inhibitors of different oligosaccharide processing enzymes in the biosynthetic pathway of *N*-glycans, the structure of the oligosaccharide can be manipulated. Failure of a macromolecule with a known altered oligosaccharide structure to reach its final destination would give clues as to the nature of the information required in intracellular traffic regulation. If the information for intracellular sorting is in the polypeptide backbone, both intracellular and cell surface forms of the unglycosylated H-2D^k should be present.

The same tunicamycin-treated and [^{35}S]methionine-labeled cells (as in Figure 5) were chased for a period up to 8 h with unlabeled methionine (Figure 6), in the presence or absence

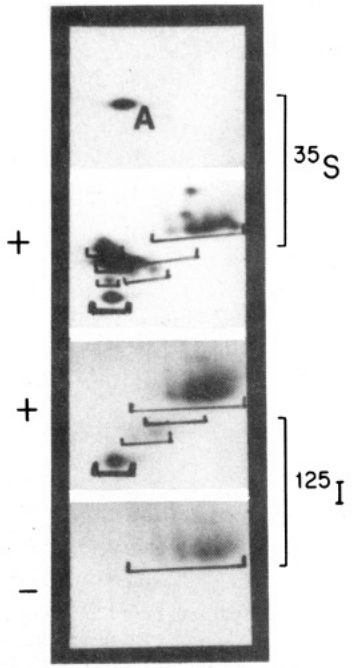

FIGURE 7: Cellular localization of H-2D^k synthesized in the presence of tunicamycin. Proteins immunoprecipitated from a tunicamycin-treated [^{35}S]methionine-labeled C3H/HeHa macrophage extract with preimmune serum (first panel) and D.32 (second panel) are shown. Also shown are the H-2D^k proteins immunoprecipitated from tunicamycin-treated ^{125}I-labeled macrophages (third panel) and untreated ^{125}I-labeled macrophages (fourth panel).

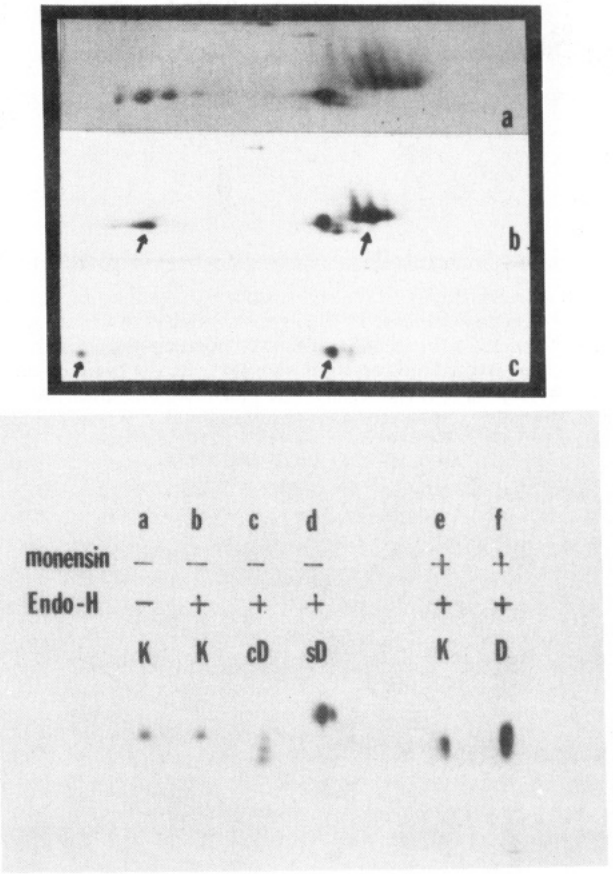

FIGURE 8: Identification of H-2K and H-2D glycoproteins with altered oligosaccharide structures. (Top) Two-dimensional electrophoretic patterns of H-2K^k and H-2D^k proteins immunoprecipitated from [^{35}S]methionine-labeled macrophages (panel a). Panels b and c represent H-2 antigens immunoprecipitated from [^{35}S]methionine-labeled macrophage treated with swainsonine and monensin, respectively. (Bottom) For the H-2 proteins immunoprecipitated from monensin-treated and [^{35}S]methionine labeled macrophages, selected spots from the two-dimensional polyacrylamide gel, indicated by arrow in panel c, were cut out and digested with endoglycosidase H as described under Materials and Methods (lanes e and f). Lanes a–d represent H-2 proteins isolated from macrophage not exposed to monensin. (+) and (–) indicate incubation of macrophages in the presence or absence of monensin, respectively, or digestion of the selected spots from the two-dimensional gel with or without endoglycosidase H.

of tunicamycin. The family of H-2D^k spots isolated from tunicamycin-treated macrophages, at the end of the chase period, was virtually identical with the control based on their relative electrophoretic mobility and molecular weight with the exception of the unglycosylated form (indicated by heavy bracket) which is now missing. Similar results were obtained whether the chase was conducted in the presence or absence of the inhibitor tunicamycin. This suggested to us that the unglycosylated polypeptides, during this chase period, are not converted to the other forms that contain one or more *N*-glycans. H-2D^k proteins immunoprecipitated from macrophages that were exposed to tunicamycin for 18 h and then iodinated also reveal the familiar ladder of spots, but there are differences between this ^{125}I-labeled family and the [^{35}S]methionine-labeled family of H-2D^k generated in the presence of tunicamycin (compare panels 2 and 3 in Figure 7). Noticeably missing in the ^{125}I-labeled family are the more basic spots (nonsialylated) containing one or more *N*-glycans seen associated with the [^{35}S]methionine labeled family. When this family of H-2D^k spots isolated from ^{125}I-labeled, tunicamycin-treated macrophages (panel 3, Figure 7) was compared to the same family of H-2D^k proteins immunoprecipitated from control ^{125}I-labeled cells (panel 4, Figure 7), in addition to the fully glycosylated and sialylated mature form of H-2D, the third panel also shows those H-2D^k forms containing one or two sialylated oligosaccharide structure of the complex type and the unglycosylated form. Since none of the glycopeptides containing nonsialylated oligosaccharide structures, presumably of the high mannose type, are labeled with ^{125}I, they are presumably not expressed on the cell surface. Accessibility of the unglycosylated H-2D^k and those H-2D^k containing one or more glycosylated and sialylated N-glycans to iodination is indicative of their presence on the cell surface (Figure 7). The results confirm and extend earlier findings showing that inhibition of glycosylation by tunicamycin did not affect the cell surface expression of unglycosylated major histocompatibility antigens (Black et al., 1981, Ploegh et al., 1981).

In the absence of the carbohydrate side chain, the H-2D and the H-2K (result not shown for H-2K) unglycosylated polypeptides are normally transported to the cell surface like their fully glycosylated and sialylated counterparts. However, the disappearance of the unglycosylated Figures at the end of the 8-h chase period (see Figures 7 and 8) together with its expression on the cell surface further support our claim that conversion of the unglycosylated H-2D^k to the other glycosylated forms did not take place. Rather, the disappearance of the unglycosylated form could result from an increase in susceptibility to proteases, rapid turnover of the protein, or shedding. In addition, the absence of this polypeptide, relative to the glycosylated polypeptides, more specifically, those polypeptides containing oligosaccharide structures of the high mannose type at the end of an 8-h chase, indicates the absence of any significant intracellular pool of unglycosylated H-2D^k. With the addition of only one N-linked oligosaccharide to the polypeptide backbone the H-2D^k glycoproteins now exhibit both cytoplasmic and cell surface forms, suggesting to us that the oligossacharide structure may play an important role in influencing the cellular distribution of this H-2D^k glycoprotein.

To address this question, inhibitors of oligosaccharide processing enzymes such as swainsonine and monensin were used to manipulate the structure of the oligosaccharide structure in an attempt to influence cellular distribution of the H-2D^k glycoproteins. Shown in Figure 8 is the two-dimensional gel electrophoresis pattern of the [^{35}S]methionine-labeled H-2D and H-2K proteins isolated from macrophages that have been exposed to swainsonine (Elbein et al., 1981) and monensin (Tartakoff, 1983). Different degrees of processing of the oligosaccharide structure, depending on the inhibitor used, can be seen (panels b and c). In each case the more acidic spots are no longer visible. In swainsonine-treated cells, terminal modifications do take place, but to a lesser extent than in the untreated cells (compare a and b). Fewer sialic acids are added, therefore, causing a change in electrophoretic mobility. This observation is consistent with the inhibitory action of swainsonine on mannosidase II which does not allow all oligosaccharide antennae to be terminally modified (Tulsiani et al., 1982; Tulsiani & Touster, 1983; Gross et al., 1983). In monensin-treated cells (panel c) the H-2 proteins do not exhibit the characteristic charge heterogeneity which has been attributed to sialylation (Le & Doyle, 1982). Rather, they migrate mainly as a single spot. The electrophoretic mobility of the H-2K^k generated in the presence of monensin is the same as the one described for cK (see Figure 2). Under similar conditions of monensin treatment H-2D^k now migrates in the same area as the smaller and more basic form of H-2D^k. Furthermore, their electrophoretic mobility is not affected by neuraminidase treatment (not shown), indicating that addition of terminal sialic acids did not occur. Similar changes in glycosylation of HLA-DR antigens in B-lymphobiastoid cells cultured in the presence of monensin has been reported (Machamer & Cressell, 1984). Following the exposure of the cells to higher concentrations of monesin, all of the [^{35}S]-methionine-labeled H-2 proteins migrate as a single spot. However, cell viability sharply decreases when higher concentrations of monensin are used. Endoglycosidase H digestion of these monensin-modified glycoproteins (indicated by arrow in panel c) does not reveal the familiar ladder of three or more spots. Rather a smear was obtained which had molecular weight ranging from 38 000 to 45 000. This suggest to use that monensin treatment generates a heterogeneous population of H-2D^k nonsialylated glycopeptides. This population would contain glycopeptides some of which are endoglycosidase H sensitive while others are endoglycosidase H resistant (Figure 9).

The H-2 glycoproteins containing altered oligosaccharide structures are normally transported to the cell surface as shown by their accessibility to cell surface iodination (Figure 9, top). They are resistant to endoglycosidase H, indicating that these cell surface forms do not contain oligosaccharide structures of the high mannose type (Figure 9, bottom). The absence of terminal sialic acid residues does not prevent these molecules from reaching the cell surface. However, the same polypeptide containing oligosaccharide structures which are endoglycosidase H sensitive consistently remains inside the cell. Processing of the high mannose oligosaccharide structure to an endoglycosidase H resistant structure is concomitant with expression on the cell surface. It appears that, at least, for the H-2D^k glycoproteins the presence of an oligosaccharide structure influences the cellular distribution of these glycoproteins and processing of the oligossacharide structure is important for cell surface expression.

Discussion

The significance of the observations described in this paper

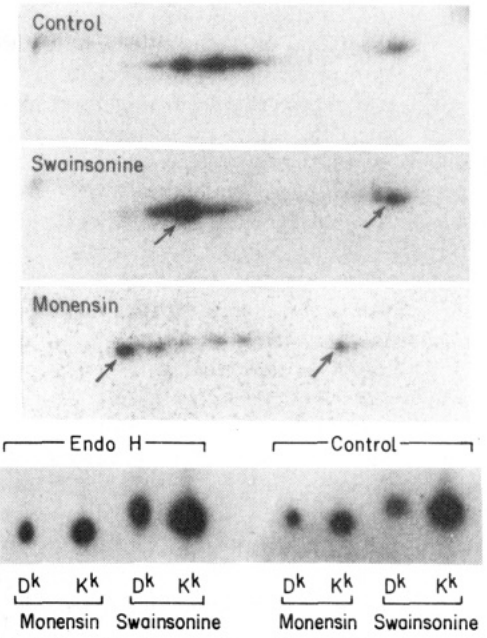

FIGURE 9: Cell surface localization of the H-2 antigens with altered oligosaccharide structures. (Top) The first panel represents the two-dimensional polyacrylamide gel electrophoresis pattern of proteins immunoprecipitated from ^{125}I-labeled macrophages. The second and third panels show the pattern of the H-2 proteins immunoprecipitated from macrophages that have been treated with swainsonine and monensin, respectively, for 15 h and then cell surface labeled with ^{125}I. (Bottom) Selected spots from the two-dimensional gel (indicated by arrows in top, second and third panel) were cut out and digested with endoglycosidase H as described under Materials and Methods.

are the following. (1) The two forms of the H-2D^k glycoproteins exist as distinct cytoplasmic and cell surface forms although both forms share similar polypeptide backbones. (2) The oligosaccharide structure of the smaller and more basic form of H-2D^k is of the high mannose type whereas the larger and more acidic form is of the complex type. (3) In the absence of the carbohydrate side chain, the H-2D^k unglycosylated polypeptide is normally transported to the cell surface like the fully glycosylated, fully sialylated counterpart. (4) The addition of only one *N*-glycan influences the cellular distribution of the H-2D^k glycoproteins. If the oligosaccharide structure is of the high mannose type, this form remains in some yet unknown intracellular compartment. Processing of this oligosaccharide structure from a high mannose type to a complex type is required for cell surface expression, but not all oligosaccharide antennae have to be terminally modified. Sialylation is not a requirement for transport to the cell surface since H-2 glycopeptides containing unsialylated endoglycosidase H resistant oligosaccharide structures can be found on the cell surface. These observations suggest that oligosaccharide structures on the H-2D^k polypeptides influence their cellular distribution.

Like all glycoproteins, the H-2 antigens are initially synthesized in the ER where they receive the core sugar $GlcNAc_2Man_9Glc_3$. In the presence of tunicamycin, which blocks the first step in the biosynthesis of $Glc_3Man_9GlcNAc_2$ pyrophosphoryldolichol, no N-linked oligosaccharide units are added to glycoproteins. The glucose residues are subsequently removed while the glycoproteins are still in the ER (Grinna & Robbins, 1979). Further processing of the oligosaccharide is thought to occur in the Golgi apparatus although mannose processing which is the next step following the removal of the glucose residues has been shown to begin before and at the arrival of these glycoproteins at the Golgi apparatus (Bischoff & Kornfeld, 1983). Swiansonine is a specific inhibitor of the

processing enzyme α-mannosidase II (Tulsiani et al., 1982) and causes the accumulation of the processing intermediate $Man_5GlcNAc_2$-Asn in the Golgi which can be further processed into a hybrid oligosaccharide containing terminal sialic acid (Saunier et al., 1982; Gross et al., 1983a,b). The specific site of action of monensin has not been clearly established. In monensin-treated cells newly synthesized glycoproteins were shown to accumulate in intracellular vacuoles that appear to be derived from the Golgi apparatus. In many cases, incomplete terminal glycosylation of these glycoproteins occurs in the presence of monensin, indicating that the action of this carboxylic ionophore is also at the level of the Golgi (Tartakoff, 1983). On the basis of these observations we believe that segregation of the two forms of the H-2D^k occurs at the pre-Golgi or Golgi level for several reasons. First, the cytoplasmic form of the H-2D^k contains oligosaccharide structures of the high mannose type. Only H-2D^k polypeptides containing complex, endoglycosidase H resistant oligosaccharide structures are expressed on the cell surface. Second, using inhibitors of oligosaccharide processing enzymes which are known to act at enzymes localized to the Golgi complex, we were able to generate H-2D^k glycoproteins containing endoglycosidase H resistant altered oligosaccharide structures which are expressed on the cell surface. These results indicate that these glycoproteins have reached the Golgi complex where terminal modification of the oligosaccharide structure occurs. Possibly the cytoplasmic form of H-2D^k accumulates in some pre-Golgi comparment or within the Golgi itself. If processing of this high mannose structure progresses normally, the H-2D^k glycoproteins follow their normal course of biosynthesis through the Golgi, where they are terminally modified, to the cell surface. Third, using tunicamycin to inhibit glycosylation, we were able to generate a heterogeneous mixture of H-2D^k molecules containing unglycosylated polypeptides and polypeptides containing one or more oligosaccharide structures. Assuming that the biosynthesis of all these molecules (unglycosylated and glycosylated) occurs randomly and their transport from the ER via the Golgi to the cell surface is not a selective process, one would expect to find all these molecules on the cell surface. Not all of these molecules are found on the cell surface. In the absence of the oligosaccharide side chains the H-2D^k polypeptides are expressed on the cell surface with no significant intracellular pool. The addition of only one N-linked oligosaccharide structure to the H-2D^k polypeptide results in the expression of cell surface and cytoplasmic forms, indicating that deposition of a significant population of H-2D^k intracellularly is influenced by the prsence of the oligosaccharide side chain. Transport of the H-2D^k is therefore a selective process. This selective transport process would appear to take place within the Golgi complex in which the oligosaccharide structure plays a very important role. It is conceivable then that the unglycosylated polypeptide would have escaped this selective process. We are presently studying this possibility and are in the process of identifying the intracellular compartment in which the cytoplasmic form of H-2D^k resides.

Alternatively there could be two different polypeptide precursors of H-2D comigrating as a single spot. These two polypeptides may contain some small differences in their primary sequence which are not detectable by limited proteolysis or by isoelectric focusing. However, this difference could be significant enough to allow the precursors to accept totally different carbohydrate side chains, giving rise to the observed subsets for the H-2D^k molecules. It is also possible that some structural characteristics which are inherent in the polypeptide backbone in addition to the oligosaccharide moiety could act as a signal to direct these two forms to their distinct cellular location. Results from the pulse–chase experiment indicate that the fully sialylated form is derived from the intracellular high mannose form. The neuraminidase experiment supports these findings. Peptide mapping by limited proteases digestion showed that both forms of H-2D^k share a strong degree of homology in the polypeptide backbone. Although we have no evidence showing differences or similarities at the amino acid sequence level between the two forms, we believe that recognition by the same antibody in addition to the criteria described above and the tunicamycin inhibition of glycosylation argue in favor of a single polypeptide precursor for both forms.

The existence of an intracellular form for H-2D^k could result from the inability of a fraction of newly synthesized H-2D^k heavy chains to bind β_2-microglobulins since this small molecule is needed for the intracellular transport and cell surface expression of histocompatibility antigens (Ploegh et al., 1979; Severinsson & Peterson, 1984). Since the intracellular forms of H-2D^k contain oligosaccharide structures of the high mannose type, one may postulate that these forms accumulate in the endoplasmic reticulum and consist of heavy chains lacking bound β_2-microglobulins. The exact intracellular localization of the cytoplasmic forms of this class 1 antigen is not known. We have no clear evidence showing that the intracellular forms of H-2D^k have bound β_2-microglobulins although in all our studies β_2-microglobulins are always present in the immunoprecipitate when either alloantisera or monoclonal antibodies to the H-2 heavy chain are used. Its presence in the immunoprecipitate following a 10-min pulse with [^{35}S]methionine in which only the smaller and more basic form of H-2D^k is labeled indicates that this form can bind β_2-microglobulin (result not shown). However, we could not determine whether all or only a fraction of the [^{35}S]-methionine-labeled H-2D heavy chains at the end of the 10-min pulse have bound β_2-microglobulins. If binding of β_2-microglobulins alone, and no other event, triggers the intracellular transport and cell surface expression of the H-2D^k glycoproteins, one would also expect the unglycosylated H-2D^k polypeptides to be present on the cell surface and to accumulate in the endoplasmic reticulum. However, the absence of any significant intracellular pool of the unglycosylated H-2D^k seems to argue, in this case, against the possible role of β_2-microglobulin in intracellular transport. Therefore, the question whether binding of β_2-microglobulins to the heavy chains is required to trigger their transport from the endoplasmic reticulum to the Golgi compartment is unresolved. We are presently looking at the possible involvement of β_2-microglobulins in the intracellular sorting of the two forms of H-2D^k and the exact intracellular localization of the cytoplasmic forms of H-2D^k.

The existence of distinct cytoplasmic and cell surface forms for the H-2D^k proteins is not unique to the k haplotype or the D locus. H-2K^d isolated from B10.D2 (Tryphonas et al., 1983) and B10.GF (Moosic et al., 1982) mice and both H-2 antigens from C57Bl/10 mice (result not shown) also exhibit distinct cell surface and cytoplasmic forms. However, of all the antigens we have studied so far, H-2K^d, H-2K^b, H-2D^b, and H-2D^k, all possess at least three N-linked oligosaccharides, and all four exhibit both cell surface and cytoplasmic forms. In contrast, the two antigens H-2K^k and H-2D^d which have only two N-linked oligosaccharides do not exhibit such characteristics. It may be that the number of N-linked oligosaccharides or its location on the polypeptide backbone may

be important in determining the cellulsar distribution of these class 1 antigens. We are also presently studying these possibilities.

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Primary Structure of the Succinyl-CoA Synthetase of *Escherichia coli*†

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ABSTRACT: The primary structure of the succinyl-CoA synthetase of *Escherichia coli* has been deduced from the nucleotide sequence of a 2451-base-pair segment of DNA containing the corresponding *sucC* (β subunit) and *sucD* (α subunit) genes. The genes are located at one end of a gene cluster that encodes several citric acid cycle enzymes: $\overleftarrow{gltA\text{-}sdhCDAB}\text{-}\overrightarrow{sucABCD}$; *gltA*, citrate synthase; *sdh*, succinate dehydrogenase; *sucA* and *sucB*, the dehydrogenase (E1) and succinyltransferase (E2) components of the 2-oxoglutarate dehydrogenase complex. The *sucC* and *sucD* genes are separated from the *sucA* and *sucB* genes by a 273-base-pair segment containing four palindromic units, but they appear to be expressed from a *sucABCD* read-through transcript that extends from the *suc* promoter to a potential ρ-independent terminator at the distal end of *sucD*. The stop codon of the *sucC* gene overlaps the *sucD* initiation codon by a single nucleotide, indicating close translational coupling of the *sucC* and *sucD* genes. The *sucC* gene comprises 1161 base pairs (388 codons, excluding the stop codon), and it encodes a polypeptide of M_r 41 390 corresponding to the β subunit of succinyl-CoA synthetase. The *sucD* gene comprises 864 base pairs (288 codons, excluding the start and stop codons), and it encodes a product of M_r 29 644, corresponding to the α subunit of succinyl-CoA synthetase. The α subunit contains a 12-residue amino acid sequence that is identical with the histidine peptide previously isolated from the phosphoenzyme. This sequence forms part of one of the two potential nucleotide binding sites detected in the α subunit.

The succinyl-CoA synthetase (SCS)[1] of *Escherichia coli* (EC 6.2.1.5) catalyzes the following reaction via three steps that involve phosphoryl enzyme and enzyme-bound succinyl phosphate as intermediates:

$$\text{succinate} + \text{CoA} + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{succinyl-CoA} + \text{ADP} + P_i$$

During aerobic metabolism it functions in the citric acid cycle coupling the hydrolysis of succinyl-CoA to the synthesis of ATP and thus represents an important site of substrate-level phosphorylation. It can also function in the other direction for anabolic purposes, and this may be particularly important for providing succinyl-CoA during anaerobic growth when the oxidative route from 2-oxoglutarate is severely repressed.

The *E. coli* enzyme has a molecular weight of 140 000 and comprises two types of subunit assembled as an $(\alpha\beta)_2$ tetramer

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[1] Abbreviations: CoA, coenzyme A; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; ODH, 2-oxoglutarate dehydrogenase; CS, citrate synthase; AK, adenylate kinase; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; IS, insertion sequence; REP, repetitive extragenic palindrome; kbp (kb in Figure 1), kilobase pair; kDa, kilodaltons. The standard single-letter code is used for amino acids.

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