E3/19K from Adenovirus 2 Is an Immunosubversive Protein that Binds to a Structural Motif Regulating the Intracellular Transport of Major Histocompatibility Complex Class I Proteins

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Summary

We have previously expressed in transgenic mice a chimeric H-2K^d/K^k protein called C31, which contains the extracellular α1 domain of K^d, whereas the rest of the molecule is of K^k origin. This molecule functions as a restriction element for alloreactive and influenza A–specific cytotoxic T lymphocytes (CTL) but is only weakly expressed at the cell surface of splenocytes. Here, we show that the low cell surface expression is the result of slow intracellular transport and processing of the C31 protein. A set of hybrid molecules between K^d and K^k were used to localize the regions in major histocompatibility complex (MHC) molecules that are important for their intracellular transport and to further localize the structures responsible for binding to the adenovirus 2 E3/19K protein. This protein appears to be an important mediator of adenovirus persistence. It acts by binding to the immaturely glycosylated forms of MHC class I proteins in the endoplasmic reticulum (ER), preventing their passage to the cell surface and thereby reducing the recognition of infected cells by virus-specific T cells. We find the surprising result that intracellular transport and E3/19K binding are controlled primarily by the first half of the second domain of K^d, thus localizing these phenomena to the five polymorphic residues in this region of the K^d protein. This result implies that the E3/19K protein may act by inhibiting peptide binding or by disrupting the oligomerization of MHC class I molecules required for transport out of the ER. Alternatively, the E3/19K protein may inhibit the function of a positively acting transport molecule necessary for cell surface expression of MHC class I molecules.

The class I antigens of the MHC consist of a family of highly polymorphic cell surface glycoproteins whose primary function is to bind and present foreign peptides to CTL (1–3). MHC class I antigens are composed of an H chain (45,000 mol wt) that contains three extracellular domains (α1, α2, and α3) and a noncovalently associated L chain (12,000 mol wt) called β_2-microglobulin (4). The crystal structure of the human class I proteins HLA-A2 (5, 6) and HLA-Aw68 (7) reveals that the α1 and α2 domains form a superdomain that is involved in peptide binding. This structure is stabilized by the α3 and β_2-microglobulin domains, which lie proximal to the plasma membrane (5, 6).

The intracellular transport of MHC class I molecules is only partially understood and appears to be regulated at several different levels. In all but exceptional cases, the transport of the MHC class I H chain out of the rough endoplasmic reticulum (RER)[1] requires association with β_2-microglobulin (8–12). Previously, this association was thought to control the rate of MHC class I passage through the cell (11, 13). However, current evidence suggests that this step may be dependent on positively acting assembly protein(s), which are constitutively expressed in B and T cell hybridomas (14), but can be induced by IFN-γ in some fibrosarcomas (15). Furthermore, recent data suggest that this step may also be controlled by the binding of peptides to the H chain, which then allows the association with β_2-microglobulin (16). At another level, MHC molecules that are highly homologous have very different transport rates, and it seems likely that

[1] *Abbreviations used in this paper:* Endo D, endoglycosidase D; Endo H, endoglycosidase H; ER, endoplasmic reticulum; RER, rough endoplasmic reticulum.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/90/12/1653/12 $2.00
Volume 172 December 1990 1653–1664

the conformation adopted by the complex between β_2-microglobulin and H chain or β_2-microglobulin, H chain, and peptides controls its rate of processing through the cell (16–18). This process could act through positively acting transport proteins or, alternatively, it could act at the level of oligomerization of MHC molecules. It follows that polymorphic structural elements in MHC proteins that do not allow ordered oligomerization can control the rate of MHC transport.

The functional implications of differential control mechanisms for MHC class I transport may be best demonstrated in responses against tumors. The lack of cell surface MHC class I proteins in some virally induced tumors may lead to the immunological nonresponsiveness of T cells (19). During adenovirus infections, a prime inhibitor of MHC expression is the E3/19K protein. In virally infected cells, this protein binds specifically in the RER to the α1 and α2 domains of MHC class I proteins and prevents cell surface expression of MHC class I proteins (20, 21). Thus, this protein can subvert immunological recognition by acting to inhibit intracellular transport of MHC molecules (22–25).

Here, we report that a recombinant MHC class I protein containing the α1 and the α2 domains from two different alleles is severely inhibited in its transport. The rate of transport is normal if the first half of the α2 domain derived from the same allele as the α1 domain is added. Therefore, this part of the α2 domain can contribute to the transport and cell surface expression of MHC antigens. We also find that similar structures are important for binding to the adenovirus protein E3/19K. These results are discussed as they relate to peptide binding, oligomerization, and *trans*-acting molecules important for MHC class I expression.

Materials and Methods

Cell Culture, Cell Labeling, Immunoprecipitation, and SDS-PAGE. Cell lines were derived and propagated as previously described (21, 26–30). Cells were maintained in DMEM containing 10% FCS.

Pulse–chase experiments were carried out essentially as described previously (29) with the following modifications. Cells to be metabolically labeled were washed twice in methionine-free medium before being incubated at 37°C with [^{35}S]methionine (1,200 Ci/mmol; Amersham International, Amersham, UK) at a concentration of 200 μCi/ml for 15 min. The cells were washed twice in warm complete medium before being resuspended in complete medium until the end of the chase time. At this point, cells were pelleted and resuspended in lysis buffer (20 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, and 40 μg/ml PMSF). To clear the lysates from the cellular debris, the tubes were spun at 14,000 *g* for 30 min. The supernatants were incubated with 3 μl of normal rabbit serum on ice for 15 min. Protein A–Sepharose (100 μl of a 1:1 buffer/Sepharose slurry) was then added, and the mixtures were incubated for an additional 60 min before being spun at 14,000 *g* for 5 min. Immunoprecipitations from the precleared lysates were carried out by incubating with 2 μl of the monoclonal ascites fluid for 45 min, followed by 45 μl of protein A–Sepharose for 45 min. The beads were washed and the proteins were analyzed by 10–15% SDS-PAGE as described previously (29).

Endoglycosidase Treatments of Immunoprecipitations. For endoglycosidase digestion, immunoprecipitates were carried out in duplicate as above. After washing in 10 mM Tris buffer, pH 7.5, immunoprecipitates were resuspended either in 25 μl of 70 mM citrate buffer, pH 5.5, alone or in 25 μl of buffer containing 2.5 mU of endoglycosidase H (Endo H) (Boehringer Mannheim Biochemicals, Indianapolis, IN). These samples were incubated for 18 h at 37°C before adding SDS sample buffer. Endoglycosidase D (Endo D) treatment was as above, except that 200 mM phosphate buffer, pH 6.5, was used.

Transfection. Transfection of the J4 and the J3 constructs into the E3/19K-expressing cell line 293.12 was carried out as described previously (28). The hygromycin B resistance gene was used to cotransfect the cells, and the cells were selected in 180 μg/ml of hygromycin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) (28).

Antibodies and Animals. All antibodies were of the IgG2a subclass, except the 1A2 antibody (anti-α-tubulin) which is IgG2b (31). The following additional antibodies were used in this study: 34-1-2s (anti-H-$2^{d,q}$) (32); 20-8-4s (anti-K^d first domain specific) (32); 11-4-1s (anti-K^k) (33); H100-27-55 (anti-H-2^k first domain specific) (34); B9.12.1 (anti-HLA) (35); W6/32 (anti-HLA-A, B, C) (36); 34-5-8s (anti-D^d) (32); 30-5-7s (anti-L^d) (32); 16-3-22 (anti-H-$2K^k$ second domain specific) (32); K9-18 (anti-H-$2K^d$ third domain specific) (26); F35-140 (anti-H-$2K^d$ second domain specific) (26). Goat anti-mouse IgG2a FITC was obtained from Southern Biotechnology Associates (Birmingham, AL).

The derivation of the C3H/He × C31 transgenic animals was described previously (29). DBA/2 (H-2^d), BALB/c (H-2^d), and C3H/He (H-2^k) were conventionally maintained.

FACS Analysis. To verify cell surface expression of the C31 antigen and H-2 antigens in transgenic, DBA/2, and C3H/He mice, FACS analysis was carried out. Mice were splenectomized, the spleens were teased into a single cell suspension, the RBC were removed by flash lysis with distilled water and were washed twice in PBS containing 0.5% (wt/vol) BSA, 20 mM NaN_3, and 20 mM Hepes, and the viability and cell number were determined by trypan blue exclusion in a hemocytometer. The splenocytes were incubated with mAbs as previously described (29), except that FITC-conjugated goat anti-mouse IgG2a was used as the second-step reagent. After labeling, the cells were fixed in 1.5% (vol/vol) *p*-formaldehyde. The fluorescent profiles were obtained by analyzing 10,000 cells in a semi-logarithmic plot on a FACS IV consort 40 program. FACS analysis was standardized using fluorescent beads from Flow Cytometry Standard Corp. (Research Triangle Park, NC).

Iodination of Splenocytes. Cell surface iodinations of mouse splenocytes were performed as described previously (37).

Other Reagents. Lactoperoxidase, restriction enzymes, and DNA-modifying enzymes were from Boehringer Mannheim Biochemicals. Protein A–Sepharose was from Pharmacia Fine Chemicals (Uppsala, Sweden). Glucose oxidase was from Sigma Chemical Co. (St. Louis, MO).

Results

We wanted to address whether the structures required for E3/19K binding to MHC molecules are the same as those controlling the intracellular transport of MHC molecules. To compare these two processes, we first examined the transport of MHC molecules and then we used this information to examine the binding of MHC class I proteins to E3/19K.

We have previously derived a C3H (H-2^k) transgenic mouse that expresses a transgenic K^d/K^k construct, called pC31, that encodes the signal sequence and α1 domain of K^d and the α2 and α3 domains, and transmembrane and cy-

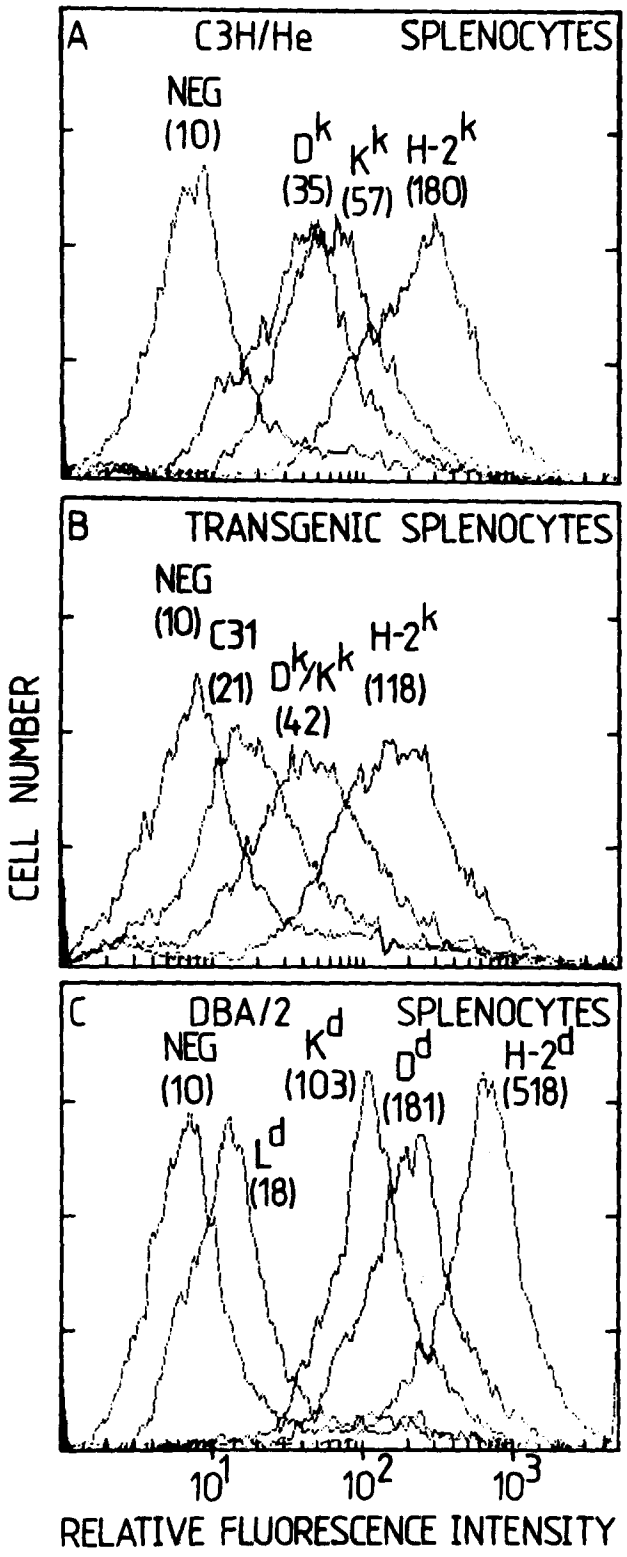

Figure 1. FACS analysis of MHC class I expression on splenocytes. The cell surface expression of MHC class I proteins on splenocytes was analyzed by flow cytometry using mAbs specific for individual MHC proteins or allotypes. Their specificities are indicated in brackets. (*A*) C3H/He (H-2^k) splenocytes were analyzed with H100-27-55 (H-2^k), 15-5-5 (H-2D^k), and 16-3-22 (H-2K^k). (*B*) C31 × C3H/He (H-2^k) splenocytes were analyzed with the same antibodies as the C3H/He cells, and in addition, the antibody 34-1-2 (H-2^d and C31) was used to detect the C31 protein. (*C*) DBA/2 splenocytes were analyzed with 34-1-2 (H-2^d), 20-8-4 (H-2K^d), 34-5-8 (H-2D^d), and 30-5-7 (H-2L^d). The negative control used in all cases was B9.12.1 (HLA specific). The mean fluorescence is shown above each profile in brackets.

toplasmic domains, of K^k. It is expressed at high levels as a messenger RNA and as a protein in most tissues. It can also function as a novel restriction element to direct specific lysis by allogenic (38) and virus-specific CTLs (29). However, the analysis of C31 cell surface expression revealed that it is expressed at lower levels than most other MHC class I proteins. Fig. 1 shows the relative amounts of MHC class I antigens expressed at the surface of splenocytes from C3H (H-2^k), DBA/2(H-2^d), and C3H/C31 transgenic mice using the FACS analysis. Note that C31 is expressed at the same level as the L^d molecule.

To investigate the low level of C31 surface expression, the rate of intracellular transport of C31 and its parental molecules, K^k and K^d, was examined. Splenocytes from C3H/C31 (265-4-1) transgenics and DBA/2 mice were pulsed for 20 min with [^{35}S]methionine and then chased for various times before lysing the cells in buffer containing 1% NP-40 and protease inhibitors. As a first approximation, long chase periods (6, 12, and 24 h) were used to determine if there was a defect in C31 processing. The K^k and C31 proteins were each immunoprecipitated from the transgenic splenocyte lysate while the K^d molecule was immunoprecipitated from the DBA/2 splenocyte lysates. In examining Fig. 2, it is apparent that C31 is not processed as quickly as the K^k and K^d molecules. The K^k molecule is completely processed from the lower molecular weight form which likely represents the RER or *cis*-Golgi form of the molecule to the mature form of the molecule by 6 h. Furthermore, the majority of the K^d molecules are processed to the *trans*-Golgi/cell surface form of the molecule in 6 h. However, the majority of the C31 molecule is not processed to the higher molecular weight cell surface form of the molecule during the 24-h chase of the experiment. Thus, C31 is much more slowly processed than either K^k or K^d. To examine this in more detail, this experiment was repeated on the transgenic splenocytes with more chase points and with treatment of duplicate samples with Endo H. The acquisition of Endo H insensitivity is a marker for the transition of a glycoprotein from the RER to the medial-Golgi cisternae (39). Fig. 3 shows that most of the K^k protein is processed to its mature form in 180 min. The K^d molecule becomes insensitive to Endo H digestion at the same time as its processed form appears, indicative of its passage from the RER to the medial-Golgi. The K^d protein is processed to its mature form in 360 min (data not shown). On the other hand, the majority of the C31 molecules do not become Endo H insensitive during the time of the chase period (Fig. 3). The C31 molecules, which do appear to become Endo H insensitive (*M*), probably represent the small percentage of the total C31 molecules made that are transported to the cell surface. However, it should be pointed out that there is ~12-fold more C31 than K^k made in transgenic splenocytes (29) and that the surface expression

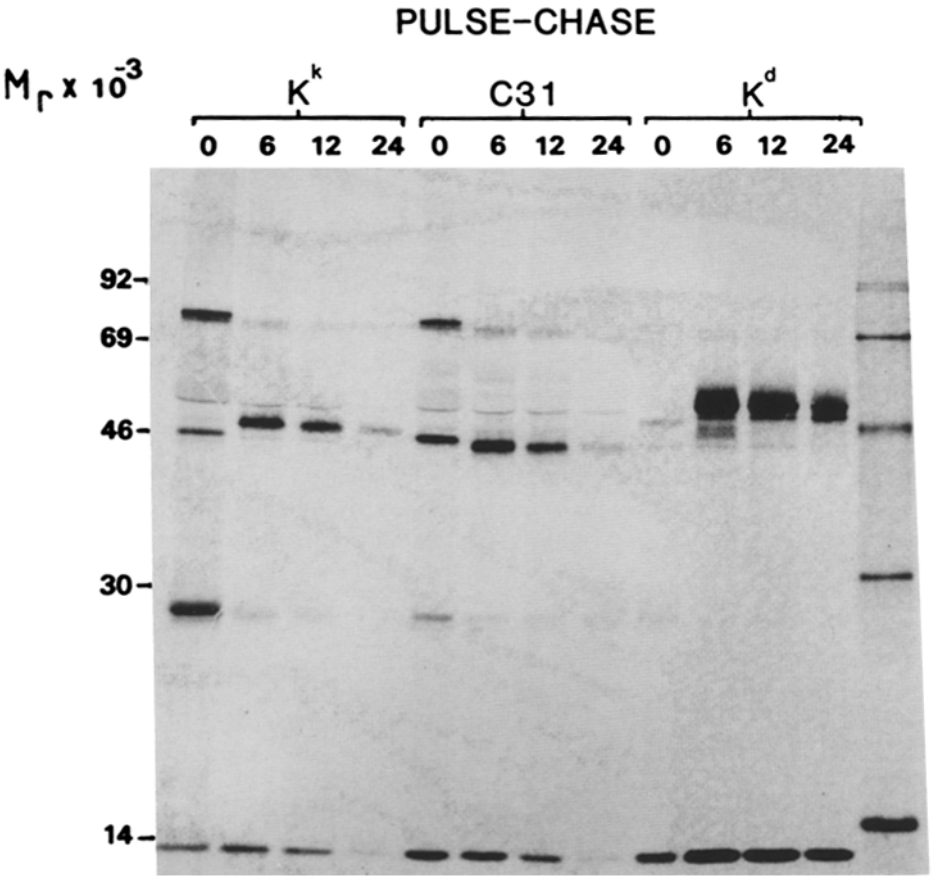

Figure 2. Pulse–chase analysis of MHC class I proteins in splenocytes. DBA/2 (H-2^d) or 253-4-1 (C31 × C3H/He) transgenic splenocytes were washed and pulsed with [^{35}S]methionine before being washed and chased with medium for the specified times in hours. The cells were harvested by lysis in 1% (wt/vol) NP-40. The antibody 11-4-1 was used to immunoprecipitate H-2K^k from the transgenic splenocyte lysates while the antibody 20-8-4 was used to immunoprecipitate C31 from the transgenic lysates and H-2K^d from the DBA/2 lysates. The molecular weight markers are shown on the right and their molecular weights ($M_r \times 10^{-3}$) on the left.

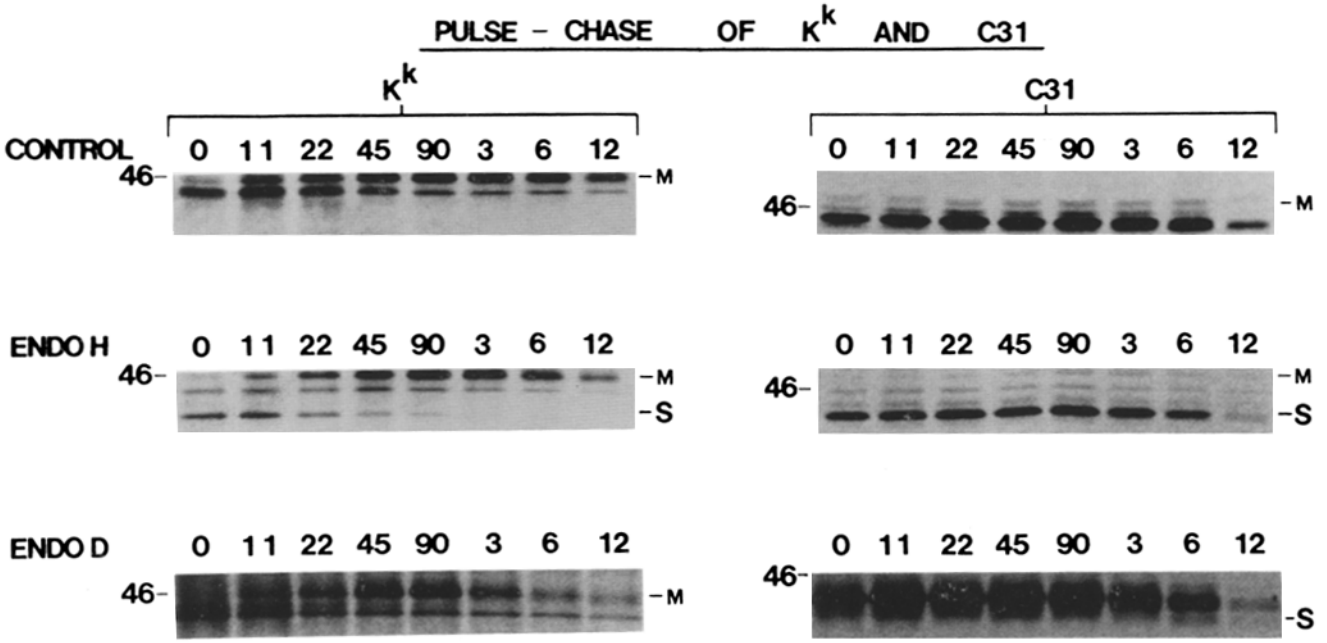

Figure 3. Pulse–chase and Endo H or Endo D digestion of K^k and C31 in transgenic splenocytes. Transgenic splenocytes of the 253-4-1 strain (C31 × H-2K^k) were pulsed with [^{35}S]methionine and then washed and chased with medium before being lysed in 1% (wt/vol) NP-40 lysis buffer at the specified times. The antibody 11-4-1 was used to immunoprecipitate the H-2K^k molecule. The antibody 20-8-4 was used to immunoprecipitate the C31 molecule. At each point the lysates were immunoprecipitated with the appropriate antibody and used as a control, or treated with Endo H or Endo D overnight. The molecular weights of the processed forms of the proteins are shown on the left and the mature form of the K^k and the C31 molecule are indicated (*M*) to the right. Forms resulting from enzymatic digestion are indicated (*S*) to the right. The migration and apparent molecular weight of OVA is shown on the left (*46*). Times are in minutes (*11*, *22*, *45*, and *90*) or in hours (*3*, *6*, and *12*).

of C31 is approximately twofold less than K^k (Fig. 1 and reference 29). Thus, the majority of the C31 molecules that are made seem not to leave the RER/*cis*-Golgi 24 h after synthesis, and this explains the discrepancy between the amount of C31 expressed at the cell surface and the total amount of C31 molecules made. To try to determine if the majority of the C31 molecule is always retained in the RER, or if some of the C31 is eventually transported to the *cis*-Golgi, a similar experiment was carried out with the transgenic splenocytes, except that Endo D was used. Acquisition of Endo D sensitivity is a marker for the transport of a glycoprotein from the RER to the *cis*-Golgi (40), and subsequent re-acquisition of Endo D insensitivity is an indication that the glycoprotein has been transported out of the *cis*-Golgi. The Endo D–sensitive $Man_5GlcNAc_2$ structure is found in the *cis*-Golgi, is only a transient intermediate of N-linked carbohydrate processing during protein transport, and is therefore not normally detectable. Fig. 3 shows that C31 becomes partially sensitive to Endo D digestion after 12 h of chasing, indicating that until this time the majority of the molecules are in the RER. No Endo D–sensitive K^k molecules can be detected and this implies that the K^k protein is transported very quickly through the *cis*-Golgi. The lower molecular weight form (*S*) is not due to degradation as it does not appear in the samples treated with buffer alone.

To demonstrate that the cell surface form of the C31 molecule has acquired complex N-linked carbohydrate structures and is normal in respects to other MHC class I proteins, transgenic and BALB/c ($H\text{-}2^d$) splenocytes were labeled at the cell surface with ^{125}I by the lactoperoxidase method and then different H-2 molecules were specifically immunoprecipitated in duplicate. One set was treated with Endo H overnight while the other set was treated with Endo H incubation buffer alone. Fig. 4 (lanes *3*, *5*, and *7*) shows that K^k, K^d, and C31 can be detected at the cell surface and that these proteins are not sensitive to Endo H (lanes *4*, *6*, and *8*). An antibody (1A2) against tubulin was used as a negative control to exclude the possibility that the iodine penetrated and labeled internal proteins (lanes *2* and *9*) and OVA, which is Endo H sensitive, was used as a control for the efficiency of the enzymatic digestion (data not shown). Therefore, the C31 expressed at the cell surface is properly processed, and the 20,000–30,000 molecules expressed at the surface of splenocytes account for the functional C31 molecules (29, 38).

It became of interest at this point to determine the structural segments that were absent in the C31 molecules but present in the K^d and K^k molecules, which allowed them to be processed and transported rapidly through the cell. To examine this phenomenon, we used intra- and interdomain hybrid genes between K^d and K^k (Fig. 5). Cell lines derived from the mouse fibroblast cell line 1T22-6 previously transfected with different hybrid genes were analyzed for cell surface expression of the specific genes using monoclonal reagents that recognize the presence of individual protein domains from K^d or K^k. The cells were examined by flow cytometry. The 20.8.4s (*A*), F35.140 (*B*), and K9.18 (*C*) antibodies were used to confirm the presence of the first, second, and third domains of K^d respectively, while the H100.27.55 (*D*), and

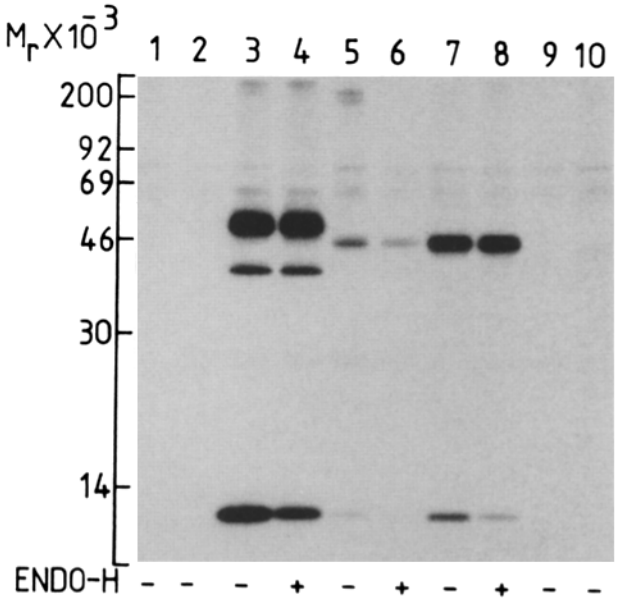

Figure 4. Lactoperoxidase-catalyzed radio iodination of BALB/c ($H\text{-}2^d$) and 253-4-1 (C31 × C3H/He) transgenic splenocytes. Splenocytes were labeled by the lactoperoxidase–glucose oxidase method. The cells were lysed in 1% (wt/vol) NP-40 and immunoprecipitates were carried out with equal TCA-precipitable counts. Lanes *1–4* were immunoprecipitated from BALB/c splenocytes and lanes *5–10* were immunoprecipitated from transgenic splenocytes. As a negative control, the material in lanes *1* and *10* were immunoprecipitated with anti-HLA (B9.12.1). As a control for the labeling of internal proteins, the material in lanes *2* and *9* was immunoprecipitated with with antitubulin (1A2); the material in lanes *3* and *4* was immunoprecipitated with anti-K^d (20-8-4); the material in lanes *5* and *6* was immunoprecipitated with anti-$H\text{-}2^d$/C31 (34-1-2); the material in lanes *7* and *8* was immunoprecipitated with anti-$H\text{-}2K^k$ (11-4-1). To determine if the cell surface forms of the H-2 class I molecules are susceptible to Endo-H digestion, duplicate immunoprecipitations were carried out which were incubated with Endo-H overnight (lanes *4*, *6*, and *8*). OVA was used as a positive control for Endo-H digestion (not shown). The 40,000–mol wt protein seen in lanes *3* and *4* is most likely the Qa protein (29).

16.3.22 (*E*) antibodies were used to respectively identify molecules containing the first or second domains of K^k. Antibody 34.1.2 (*G*) was used in this experiment to define the endogenous $H\text{-}2^q$ molecules expressed in the untransfected 1T22-6 cells. With these reagents we were able to demonstrate the expression of individual domains of the hybrid molecules in each of the transfected 1T22-6 cell lines. More than this, we find that there is a striking correlation between the domain structures and the cell surface expression of the particular molecule. Consistent with the results on the transgenic splenocytes, the C31 molecule is expressed at much lower levels than either the K^d or K^k at the surface of the 1T22-6 cell. This result was consistently reproduced with five individually derived C31 1T22-6 transfectants (data not shown). However, the molecules that contain, in addition to the first domain of K^d, the second (C33) or the second and third (C32) domains of K^d are expressed at levels similar to the K^d molecule. The intradomain hybrid molecule J4, which contains the first domain of K^d and the first half of the second domain of K^d, is expressed at the same level as the

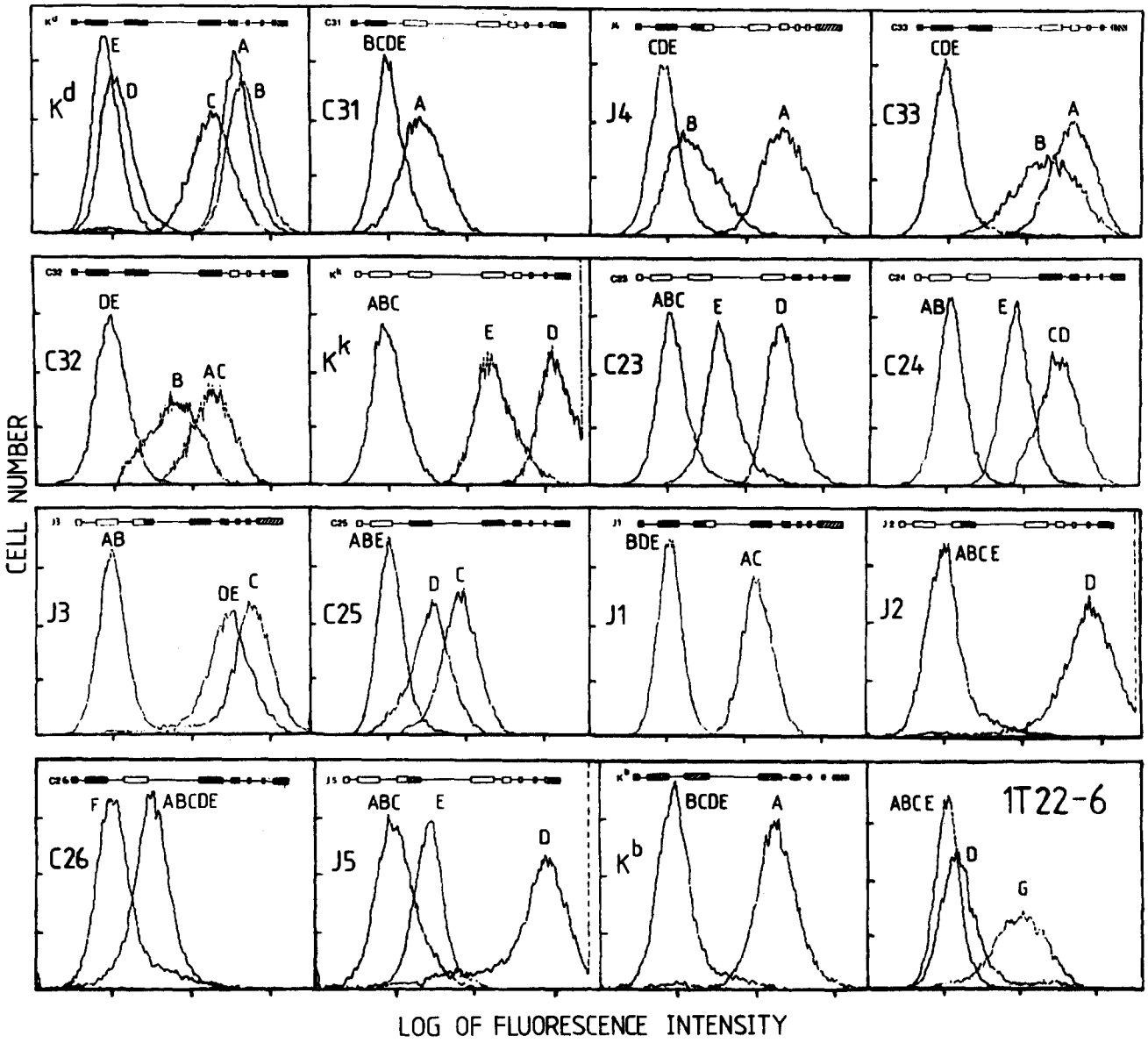

Figure 5. Comparison of the cell surface expression of the H-2K^d/K^k hybrid genes expressed in 1T22-6 cell lines by flow cytometry. The cells were incubated with (*A*) 20-8-4 (anti-K^d, domain one), (*B*) F35-140 (anti-K^d, domain two), (*C*) K9-18 (anti-K^d, domain three), (*D*) H100-27-55 (anti-K^k, domain one), (*E*) 16-3-22 (anti-K^k, domain two), (*F*) no first antibody, and (*G*) 34-1-2 (anti-H-2^q endogenously expressed by 1T22-6 cells). The binding of the first layer of antibody was determined with FITC-conjugated goat anti-mouse Ig and quantified with fluorescent microbeads. The construct expressed in each of the transfectants is depicted at the top of each of the panels.

K^d protein. The results with this construct localize the segment responsible for complementation of C31 cell surface expression to the first half of the second domain of K^d. To show that the third domain does not contribute to this phenomenon, the C26 protein was also analyzed. This molecule has the first and third domains of K^d and the second domain of K^k, and like the C31 molecule, is also expressed at low levels at the cell surface. Thus, the low level expression can be linked entirely to elements in the first domain and the first half of the second domain of K^d.

To try to generalize this finding, the reciprocal constructs containing the K^k domains in place of the K^d domains were analyzed. The C23 and C24, which contain, respectively, the first three domains of K^k or the first two domains of K^k, are expressed at about the same level as K^k, while the C25 molecule, which contains the first domain of K^k and the second and third domains of K^d, is expressed at slightly lower levels than the K^k protein but not as low as the C31 molecule. Again in the case of this molecule, the addition of the first half of the second domain of K^k appears to increase the expression of the C25 molecule. The J2 molecule and the J5 molecule, which contain the corresponding K^d or K^b segments in the second half of the second domain of K^k, demonstrate that this region does not contribute to the

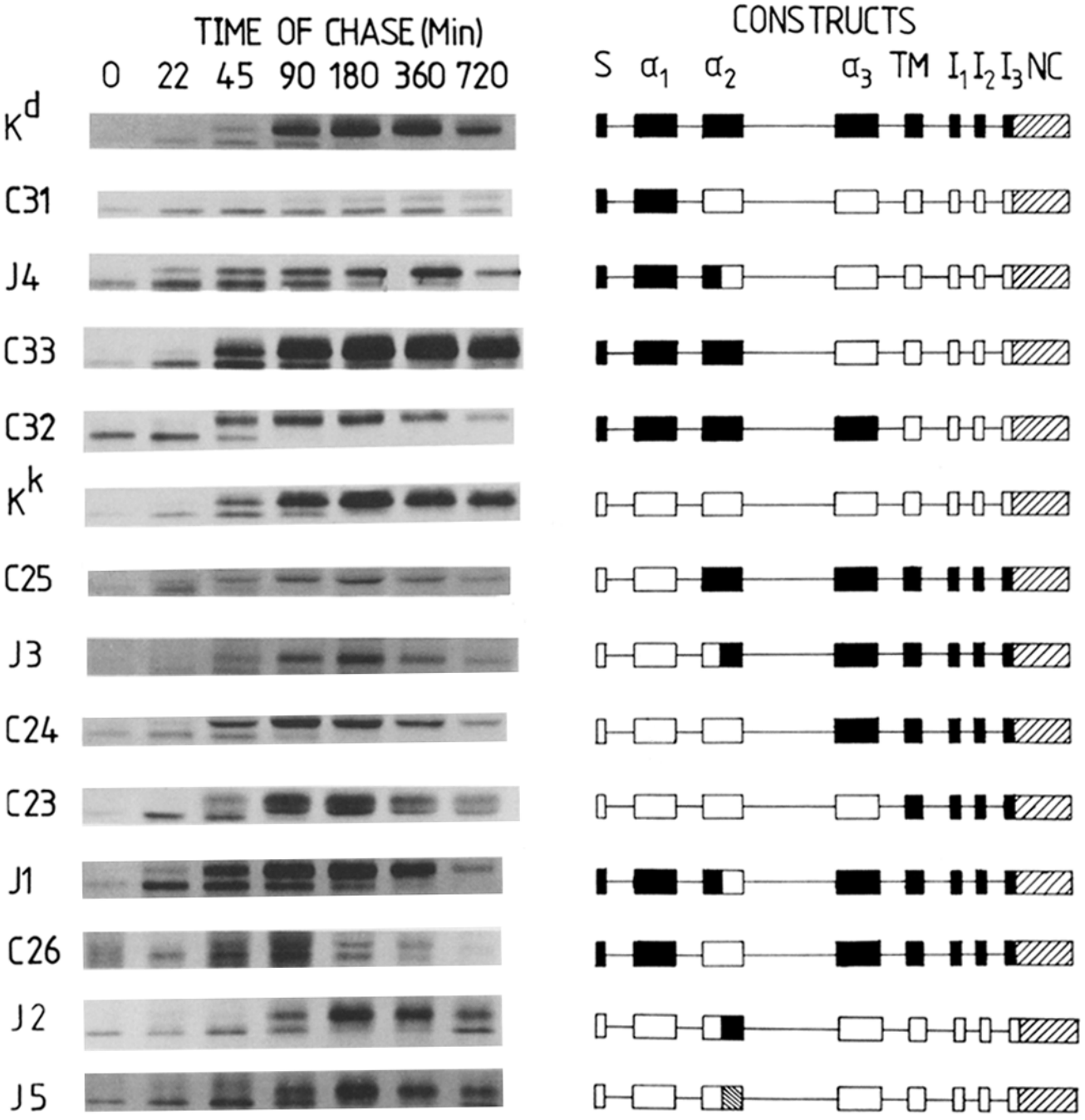

Figure 6. Localization of the segment of K^d that complements C31 transport and processing. Transfected 1T22-6 cells expressing the H-$2K^d/K^k$ hybrid molecules were pulsed with [^{35}S]methionine and then washed and incubated in medium. The cells were lysed in 1% (wt/vol) NP-40 at the specified times. The antibody 20-8-4 was used to immunoprecipitate molecules containing the first domain of K^d, and H100-27-55 was used to immunoprecipitate molecules containing the first domain of K^k.

cell surface expression of the K^k molecule. Thus, the first domain together with the first part of the second domain of H-$2K^d$, and to a lesser extent H-$2K^k$, contain amino acids that strongly influence their cell surface expression.

To determine whether the cell surface expression of the C31 and the C25 proteins are related to their transport, the rate of intracellular processing of hybrid proteins was examined in pulse–chase experiments. Fig. 6 shows that in 1T22-6 cells the mature form of the K^d molecule appears at 45 min and it is completely processed after 3 h ($t_{1/2}$ = 50 min), while the mature form of the K^k molecule appears after 22 min and is completely processed at 3 h ($t_{1/2}$ = 35 min). These results parallel those in the splenocytes pulse–chase experiments where the K^k molecule is processed slightly faster

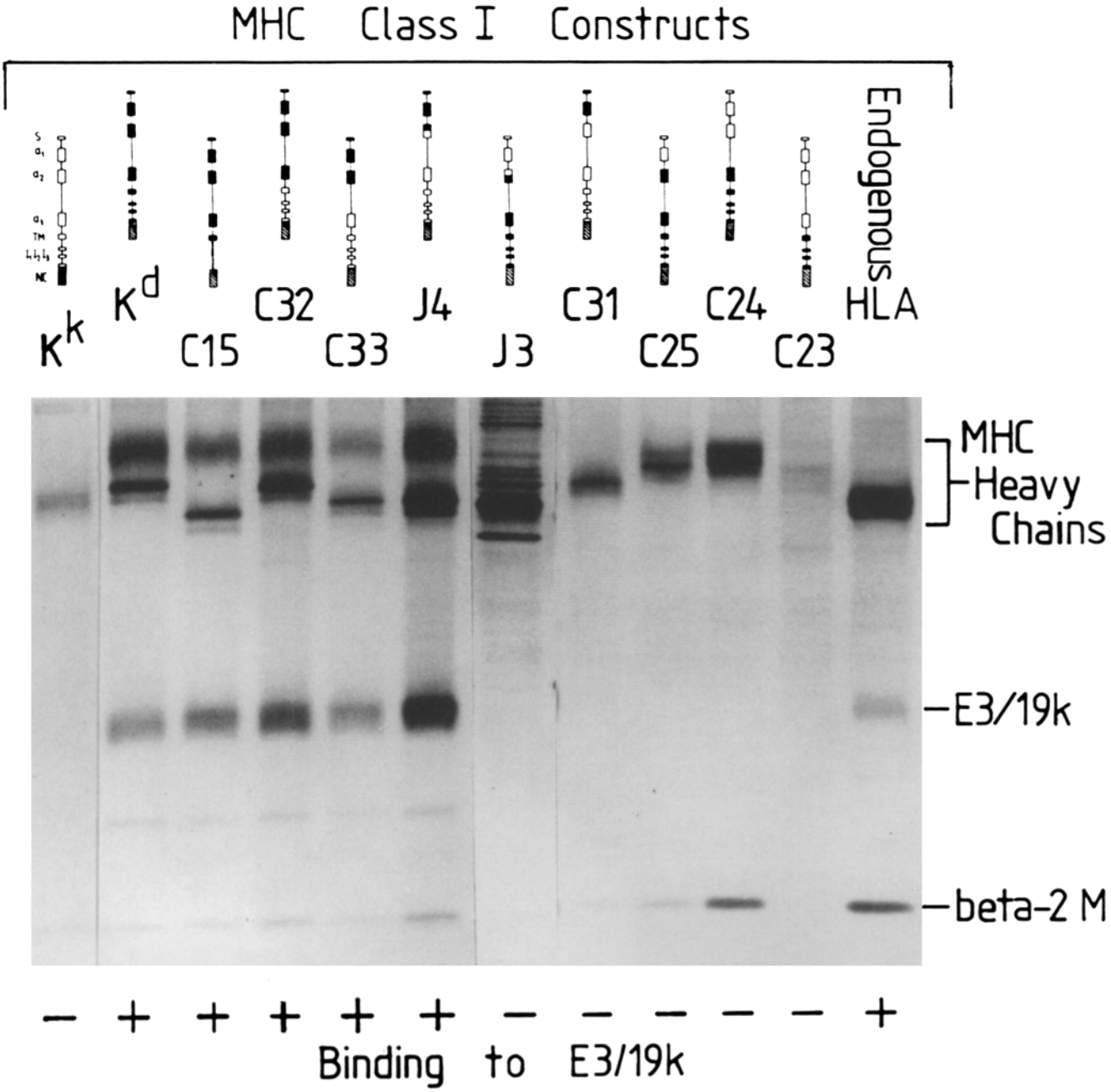

Figure 7. Characterization of the structure within K^d responsible for the binding to the immunosubversive E3/19K protein of adenovirus 2. 293 cells previously transfected with the gene encoding the E3/19K protein were supertransfected with the hybrid K^d/K^k genes. Cells expressing the hybrid genes were selected and then were labeled with [^{35}S]methionine for 20 min, washed, and then chased with medium for 1.5 h before being lysed in 1% (wt/vol) NP-40. Proteins containing the first domain of K^d were immunoprecipitated with the 34-1-2 antibody, while proteins containing the first domain of K^k were immunoprecipitated with H100-27-55. W6/32 was used to immunoprecipitate HLA class I proteins from 293 cells expressing the E3/19K protein. The constructs analyzed are depicted at the top of each lane. The ability to bind E3/19K is shown at the bottom of the figure.

than the K^d molecule. The mature form of the C31 protein can be visualized after 22 min of chasing; however, the processing of the C31 molecule is not completed at 720 min ($t_{1/2}$ = 10 h), and thus the processing of the C31 molecule may take longer than the division time of the 1T22-6 cells. It is intriguing that the overall rate of processing of these MHC class I proteins is faster in the 1T22-6 cell line than in splenocytes.

If one compares the processing of the J4 construct with that of the C31 protein, it is evident that the J4 construct ($t_{1/2}$ = 55 min) is processed much more rapidly than the C31 molecule. Similarly, the C33 ($t_{1/2}$ = 50 min) and C32

($t_{1/2}$ = 40 min) molecules are processed at approximately the same rate as the K^d protein. This information suggests that the segment that is required for more rapid transport of C31 through a cell is present in the first half of the second domain of the K^d molecule. To address whether the third domain can interact with the first domain to allow transport, the C26 protein was analyzed. This protein is processed at approximately the same rate as the C31 protein ($t_{1/2}$ = 6 h) and shows that the third domain has only a minor influence on processing. Finally, the J1 construct ($t_{1/2}$ = 45 min), which contains the second half of the second domain from K^k, while the rest of the molecule comes from K^d, is processed normally and re-emphasizes the localization of the segments that speed processing of the C31 protein to the first half of the second domain. Therefore, the cell surface levels of the hybrid proteins correlate with the rate of processing, suggesting that the major contributor to the amount of C31 expressed at the cell surface is the rate of processing. Furthermore, we localize the structure that controls the rate of processing to a combinatorial association between the first domain of K^d and the first half of the second domain.

In the case of the C25 molecule we obtain a different result. The C25 protein appears to be rapidly processed; the mature form of the molecule appears in 90 min. The J3, C24, and C23 proteins, which contain progressively more K^k residues, are also processed quickly. The internal expression of C25 is essentially the same as other hybrid proteins (data not shown) and does not, therefore, account for the difference in cell surface expression. However, the lower C25 expression at the cell surface may be due to slower passage from the *trans*-Golgi to the cell surface or, in an alteration in the affinity of the antibodies we are using to analyze the C25 molecule at the cell surface.

We previously showed that the E3/19K protein of adenovirus 2 inhibits the transport of some MHC molecules to the cell surface by specifically interacting with them in the RER (20, 21). It has been shown that MHC class I molecules must present foreign antigens at the plasma membrane to T cells in order to generate an immune response against the foreign antigen. Thus, the inhibition of MHC class I expression at the cell surface represents an interesting viral mechanism for subverting the immune response and possibly for establishing viral persistence. In further studies, we demonstrated that the K^d molecule binds the E3/19K protein, while the K^k protein does not. The structures required for this interaction mapped to the first 180 amino acids (α1 and α2 domains) in the K^d molecule. We investigated whether the same segment of the α1 and α2 domains which control K^d transport are also involved in E3/19K-induced transport inhibition. Therefore, the J4 and the J3 molecules were transfected into the human embryonic kidney cell line 293.12, which had previously been transfected with the E3/19K gene and the neomycin resistance marker. We used the hygromycin resistance gene for cotransfection with the J3 and J4 genes to select for clones harboring the new MHC genes. Individual clones were grown up to be analyzed with other previously derived chimeric gene transfectants. These cell lines were pulsed for 20 min with [^{35}S]methionine and then chased for 90 min. mAb specific for the transfected MHC class I protein were used to detect binding of the adenovirus protein to the MHC protein via coimmunoprecipitation. Fig. 7 shows that the K^d, C33, and C32—but not C31 or K^k—bind the E3/19K protein, and that those molecules that bind the E3/19K protein are not processed to their mature form 1.5 h after the pulse. The J3 molecule does not bind the E3/19K protein and its transport is not inhibited. Thus, the second half of the second domain of K^d is not sufficient to bind the E3/19K protein. On the other hand, the J4 molecule binds the E3/19K protein, and its processing is inhibited. These data localize the segment that binds the E3/19K protein to the first half of the second domain of the K^d molecule or to a combinatorial region comprising the first domain of K^d and the first half of the second domain of K^d. Thus, the same segment that controls transport of the K^d molecule out of the RER also controls the binding to the E3/19K protein which itself inhibits passage out of the RER.

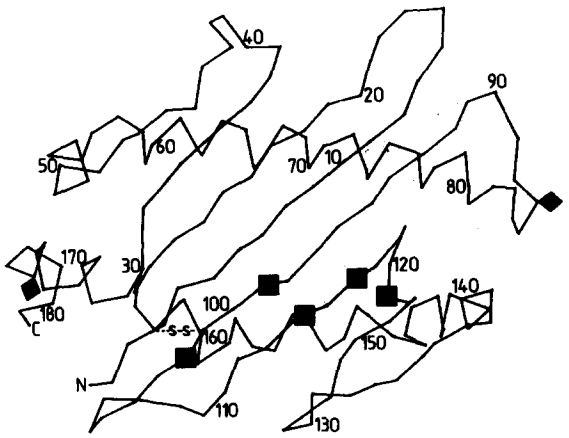

Figure 8. Colocalization of amino acids controlling transport and E3/19K binding. The α carbon coordinates of the first and second domains of the MHC protein HLA-A2 are depicted. The black squares indicate the residues that both determine the rate of intracellular transport of the H-2 K^d molecule and dictate its binding to the immunosubversive E3/19K protein of adenovirus 2. The diamonds indicate the sites of N-glycosylation.

Discussion

Plasma membrane proteins that are synthesized in the RER must be transported via the exocytotic pathway to the cell surface. The amount of protein on the cell surface may be determined by a number of factors, such as the rate of synthesis, the rate of transport out of and back into the cell, and ultimately by the rate of degradation. The rate of transport of MHC class I molecules through the cell appears to be primarily controlled by the rate of egress from the RER to the *cis*-Golgi region (17, 18). The noncovalent interaction of the class I H chain with the soluble protein β_2-microglobulin presumably alters the conformation of the molecule (12) but is not the only factor that contributes to the rate of transport. Recent data suggest that the binding of β_2-microglobulin to H chains in the RER is not spontaneous but ap-

pears to be under the control of one or several factors. The description of these factors was made possible by the generation of cell lines that did not allow the association of synthesized nascent chains of β_2-microglobulin and H chains in the RER (14, 15). In one case, the association could be induced by IFN-γ (15) while, in another, association was achieved by somatic cell hybridization with a wild-type cell line (14). In another intriguing study, the association could be induced with peptides that are known to associate with the MHC class I molecule during viral infection (16). Thus, this study suggested that a peptide specifically induced the expression of its own restriction element. Thus, it is clear from this and other studies that the association and subsequent transport out of the RER is under the control of positively acting factor(s). The final level of control of MHC transport from the RER is the subject of experiments presented here and is an extension of other work, which describes that even after binding β_2-microglobulin, highly homologous MHC class I proteins differ in their rate of transport to the cell surface (17). We found that the majority of C31 molecules that bound to β_2-microglobulin are transported at a very slow rate from the RER to the *cis*-Golgi. During the first 12–24 h, most of the C31 protein in splenocytes is Endo H sensitive and it takes 12 h for the majority of the C31 molecules to acquire Endo D sensitivity. These markers for protein transport and processing place the C31 molecule in the RER or the *cis*-Golgi 12 h after synthesis. Combining the results of Williams et al. (18) with our own, we suggest that the C31 protein is not transported rapidly because it lacks the correct conformation required for transport. At present we can only speculate on what disturbs the transport-competent structures in molecules like C31. We do not think that binding of β_2-microglobulin is reduced, as coimmunoprecipitation of β_2-microglobulin seems to be in the normal range (Fig. 2). We favor the idea that discrete structures in the $\alpha1/\alpha2$ superdomain are important for transport. This is in agreement with the studies of Williams et al. (17), which show that different MHC molecules have different rates of transport, indicating that transport is controlled by polymorphic amino acid residues. In light of the results of Townsend et al. (16), we suggest that in our system, the mixed $\alpha1$ (K^d)/$\alpha2$ (K^k) domain is able to bind β_2-microglobulin, but it is unable to bind a large number of peptides, which results in a reduction of transport and cell surface expression. However, we can not rule out the direct effect on oligomerization and/or transport. We should note here that the 20,000–30,000 molecules of C31 that we can detect at the cell surface by saturated binding assay seem to be normal, as evidenced by their higher molecular weight form, their insensitivity to Endo H after cell surface radio-iodination, and their demonstrated function in CTL assays (29, 38).

We proceeded to localize the segment of the K^d protein that contributes to faster processing to the C31 protein. The rate of intracellular processing of the K^d-like constructs (NH_2-terminal domain derived from K^d) and the K^k-like constructs (amino acid domains derived from K^k) were analyzed by pulse–chase experiments in 1T22-6 cells to determine whether their rate of processing is consistent with the cell surface expression of the hybrid proteins. Fig. 6 shows that the processing of the C31 molecules is ~15-fold slower than most of the other molecules tested. For most molecules, the time point at which 50% of the molecules are in their higher molecular weight forms is between 45 and 90 min, whereas for the C26 molecule it takes ~6 h and for C31 it takes ~10 h. In comparison with the C31 molecule, the J4 protein is rapidly processed. This corroborates the cell surface analysis and again suggests that the first half of the second domain of the K^d molecule contains residues that are important for transport out of the endoplasmic reticulum (ER). The C26 molecule is transported slowly through the cell, and thus the third domain and the rest of the molecule does not contribute significantly to the rate of processing. Analysis of the J1 protein that contains only the second half of the second domain of K^k while the rest of the molecule is from the K^d protein confirms the above statement. Accordingly, the J1 protein is transported at a similar rate to the K^d protein. Thus, we have identified a structure that influences the rate of K^d processing within the first half of the second domain. The same stretch of amino acids does not appear to be important for the efficient transport of the K^k protein out of the ER; however, it may be important for the transport of the K^k molecule from the *trans*-Golgi to the cell surface (see Fig. 5).

It was then of particular interest to test whether the structural elements identified above are also involved in transport inhibition of MHC antigens in cells expressing the adenovirus E3/19K protein. We have previously reported that the K^d, but not the K^k protein, binds the E3/19K protein from adenovirus 2 and, more specifically, that the first two domains of K^d were responsible for binding this viral protein. Intriguingly, the C31 protein does not bind (18). We transfected the J4 and the J3 molecules into the human 293.12 cell line that had previously been transfected with the E3/19K gene (21). J4- or J3-positive clones were expanded and analyzed for coimmunoprecipitation of the E3/19K protein with either of these hybrid proteins (Fig. 7). We compared the binding of these hybrid genes with those previously transfected. We found that the J4 construct bound very efficiently to the E3/19K protein but that the J3 protein did not. The J3 construct contains the first domain and half of the second domain of K^k while the rest of the molecule is from K^d. The binding of the J4 construct suggests that in addition to protein transport, the polymorphic residues in the first half of the second domain of the K^d also contribute to the binding of the E3/19K molecule. We suggest that this finding is not coincidental. We speculate that we have localized the structure in K^d that is either required for oligomerization or is recognized by a positively acting transport protein. Symmetrical packing into transport vesicles or oligomerization may be required for efficient transport out of the ER. It is possible that we have identified the structure in K^d largely responsible for this phenomenon. The function of the E3/19K protein may be to bind to this structure and disrupt assembly into transport vesicles. The major alternative is that this structure is recognized by a protein that actively transports MHC molecules out of the ER. The C31 protein, lacking this struc-

ture, would not be recognized by the transport protein and would not be efficiently transported. The function of the E3/19K protein would then be to directly compete with the transport protein for binding to the transport facilitation structure. At present we cannot differentiate between these two possibilities; however, recent work by Livingstone et al., (41) favors the latter model. They demonstrate the existence of an MHC-linked gene, called *cim*, which is required for efficient transport to the cell surface of rat MHC class I molecules after binding to β_2-microglobulin in the ER.

The crystal structures of the HLA-A2 and AW68 molecules have been determined (5–7). Here, we assume that other MHC class I proteins will have a similar structure, and we also assume that the structure of the ER form of the MHC class I protein is not grossly different from the papain fragment. This information can be used to analyze the structure in the second domain that contributes to transport and E3/19K binding. The residues that are polymorphic between the C31 protein and the J4 protein (residues 99, 102, 114, 116, and 121) are localized within the crystal structure of HLA-A2 in Fig. 8. The amino acids at positions 99, 114, and 116 point into the putative peptide binding pocket, while the amino acid at position 102 is localized below the α helix of the second domain and position 121 is on a loop between β-pleated strand β_2 and β_3. Amino acids 99 and 102 make interdomain contacts with the α1 domain, and disruption of these interactions may disturb the gross structure of the superdomain. On the other hand, amino acids 116 and 121 contact β_2-microglobulin, and it is conceivable that these residues may also affect the overall structure of MHC class I proteins while leaving binding to β_2-microglobulin unaffected. Finally, if E3/19K directly competes with peptides at the binding cleft or if peptides can alter the intracellular transport of MHC molecules as suggested by Townsend et al. (16), residues that affect peptide binding could influence both of these processes. Thus, residues 99, 114, and 116 are candidates that could control both E3/19K binding and MHC transport. Changing single amino acids in this region by site-directed mutagenesis should allow us to identify the amino acid(s) important for MHC transport and E3/19K binding and help to elucidate the mechanisms involved.

We thank Dr. Sune Kvist for his continued support and encouragement. We would also like to thank Drs. R. F. Petterssen, J. Saraste, R. Persson, R. Gabathuler, B. Dalhof, F. Levy, R. McMaster, and F. Tufaro for stimulating discussions; Dr. D. Wiley for access to the alpha-carbon coordinates of the HLA-A2 protein, and Drs. U. Ruther and E. Wagner for helping to generate the C31 transgenic mice.

This work was supported by grants to W. A. Jefferies by the National Cancer Institute of Canada and by the British Columbia Health Care Research Foundation.

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Received for publication 9 July 1990 and in revised form 7 September 1990.

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