Clonal Variation in Cell Surface Display of an H-2 Protein Lacking a Cytoplasmic Tail

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Abstract. Truncated variants of the gene encoding H-2L^d, an integral membrane protein encoded by the major histocompatibility complex, were constructed by in vitro mutagenesis to elucidate the function of charged amino acids found on the cytoplasmic side of the transmembrane (TM) region. Analysis of cloned L cells transfected with these genes shows that the seven amino acids following the TM segment, four of which are basic, enhance the cell surface expression of H-2L^d protein but are not required for it. However, some clones do not express a tailless H-2L^d protein on the cell surface but express it intracellularly where it has a long half-life. Turnover measurements on cell surface H-2L^d proteins suggest that the basic residues following the TM segment are not a "stop transfer" sequence (Blobel, G., 1980, *Proc. Natl. Acad. Sci. USA.*, 77:1496–1500) which anchors the H-2L^d protein in the membrane. Pulse-chase and endoglycosidase H sensitivity studies show that H-2L^d proteins lacking some or all of the basic residues and H-2L^d proteins which have a full-length cytoplasmic tail are processed with different kinetics. These results suggest an involvement of the membrane-proximal region of the cytoplasmic tail in the intracellular transport of H-2L^d. We further suggest that the L cell clones which do and do not express a tailless H-2L^d protein on the cell surface differ in the ability to transport a tailless integral membrane protein to the cell surface.

THE identification of the structural features that are required for proper cellular localization of proteins is one of the major unsolved problems of cell biology (Blobel, 1980; Sabatini et al., 1982). In the case of integral membrane proteins, it is known that initial sorting occurs during synthesis, at which time the nascent protein is inserted into into the lumen of the endoplasmic reticulum (ER)[1] (Blobel and Dobberstein, 1975*a, b*). This occurs as a consequence of the interaction of the signal sequence on the polypeptide chain with the signal recognition particle (Walter and Blobel, 1980). Secretory (Blobel and Dobberstein, 1975*a*) and lysosomal (Erickson and Blobel, 1979) proteins are also sorted into the ER by this mechanism, and further compartmentalization occurs during the subsequent processing of these different types of proteins (Strous and Lodish, 1980; Gumbiner and Kelly, 1982). In addition to the signal sequence on the precursor protein, another structural feature typical of integral membrane proteins is the highly hydrophobic membrane-spanning (transmembrane or TM) region which is necessary and sufficient for insertion of a protein into membranes in vivo and in vitro (Boeke and Model, 1982; Davis et al., 1985; Davis and Model, 1985; Gething and Sambrook, 1982; Rose and Bergmann, 1982, 1983; Sveda et al., 1982; Yost et al., 1983). It would appear, however, that there are additional structural requirements for the transport of an integral membrane protein to the cell surface, since attachment of the TM region of vesicular stomatitis virus (VSV) G protein to rat growth hormone results in the transport of the fusion gene product to the Golgi apparatus, but not to the cell surface of transfected fibroblasts (Guan and Rose, 1984). Moreover, in cells having specialized plasma membranes, such as polarized epithelial cells, the preferential localization of certain integral membrane proteins to particular regions of the cell surface might be achieved by cellular mechanisms which include recognition of specific structural features on the glycoprotein itself. For example, polarized African green monkey kidney cells infected with recombinant SV40 viruses containing the influenza hemagglutinin gene preferentially sort the hemagglutinin to the apical surface on which it is normally expressed when the cells are infected with influenza virus (Roth et al., 1983).

Class I histocompatibility antigens are integral membrane glycoproteins (Springer and Strominger, 1976) found on the surfaces of almost all nucleated mammalian cells, where they serve as self-recognition structures for T lymphocytes during cellular immune responses (Zinkernagel and Doherty, 1979). The class I glycoprotein (45,000 daltons) is noncovalently associated via its extracellular portion with a nonpolymorphic

[1] *Abbreviations used in this paper:* Endo H, endoglycosidase H; ER, endoplasmic reticulum; FCS, fetal calf serum; β_2M, β_2-microglobulin; mAb, monoclonal antibody; RIA, radioimmunoassay; SSPE, 0.18 M NaCl, 1 mM EDTA; 10 mM $NaPO_4$ buffer, pH 7.0 ; tk, thymidine kinase; TM, transmembrane; VSV, vesicular stomatitis virus.

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light chain, β_2-microglobulin (β_2 M) (Cresswell et al., 1974), which is not itself an integral membrane protein (Ploegh et al., 1981).

Five distinct regions of the class I histocompatibility antigen have been identified by structural and functional studies (Ploegh et al., 1981; Nathenson et al., 1981). The three external regions, which are of immunological importance, are followed by a TM region of approximately 25 amino acids and an approximately 30-amino acid cytoplasmic tail, most of which can be removed without affecting the cell surface expression of a functional protein (Zuniga et al., 1983; Murre et al., 1984).

Since the biosynthesis of class I histocompatibility molecules has been extensively studied (Krangel et al., 1979; Dobberstein et al., 1979), and since the cloned genes for a number of these proteins are available (Hood et al., 1983), these proteins provide a good system for the identification of sequences important for the transport, membrane integration, and cell surface expression of an integral membrane protein. Using the technique of in vitro mutagenesis, we have constructed a series of truncated variants of a class I gene, the H-$2L^d$ gene of the BALB/c mouse, and have generated stable transformant mouse L cell lines expressing these mutant genes. Here we describe the properties of cloned cell lines which have received H-$2L^d$ genes encoding proteins with truncated cytoplasmic tails which differ in the number and nature of charged residues.

Materials and Methods

Plasmids and Cloned Genes

Plasmids 27.5.27 (the H-$2L^d$ genomic clone), p2001, p1800-2, and pTK5 have been described previously (Zuniga et al., 1983). Plasmids were grown in *Escherichia coli* strains 490A, HB101, or MC1061.

Synthetic Oligonucleotides

Oligonucleotides were synthesized and purified as described.[2] The complementary oligonucleotides for the BAL31/linker mutagenesis strategy were phosphorylated with γ^{32}-P-ATP and polynucleotide kinase, mixed, heated to 68°C for 5 min, made to 0.1 M NaCl, 1 mM EDTA, and allowed to anneal at 50°C for several hours. These were then ligated to the p958 BAL31 deletion products (see below) with T4 DNA ligase (Zuniga et al., 1983).

In Vitro Mutagenesis of the H-$2L^d$ Gene Subgene Fragments Used to Reconstruct Mutant Genes

BAL31 deletion mutagenesis of the H-$2L^d$ gene was performed as described previously (Zuniga et al., 1983). The substrate for initial mutagenesis was p1800-2 (Goodenow et al., 1983; Zuniga et al., 1983). After linearization by cleavage with Sac I and incubation with BAL31 nuclease, the digestion products were ligated to phosphorylated Cla I linkers (New England Biolabs, Beverly, MA), and then digested to completion with Cla I. This resulted in two populations of BAL31 deletion fragments. One contains most of pBR322 and the Bam HI-Cla I part of the 1800-2 insert bearing exons 4 and 5 and flanking sequences. This fragment was recircularized by incubation with T4 DNA ligase. The other Cla I product contains 356 base pairs of pBR322 sequence and the 3′ end of the 27.5.27 gene, including the 3′ untranslated region. This latter population of fragments was cloned into the Cla I site of pBR322. The two resulting populations of plasmid molecules were transformed into *E. coli* 490A, and individual colonies were grown and characterized by restriction enzyme analysis. Representative clones were then grown and sequenced by the chemical method of Maxam and Gilbert (1977). Clone p958 extends from the Bam HI site in the third intron, includes exons 4 and 5, and extends to nucleotide 2468 (the second nucleotide of exon 6). All subsequent mutagenesis (see below) was performed on p958, and then intact H-$2L^d$ genes were generated by a strategy similar to that illustrated in Fig. 1 of Zuniga et al. (1983). The first step of reconstruction of an intact gene used plasmid p903, which contains exon 8 (3′ untranslated region of 27.5.27) and includes 125 base pairs of upstream intervening sequence. p903 is the second kind of clone derived from the BAL31 mutagenesis of p1800-2 described above. The 3′ half of the gene was reconstructed by cloning the Cla I insert of p903 into the Cla I site of the mutant derivative of p958 (see below). Clones in the correct orientation were identified by restriction enzyme analysis. The mutant 3′ half genes are contained within a 2.2-kilobase pair Bam HI fragment which in all cases was subcloned into the Bam HI site of p2001, which contains exons 1–3 (Zuniga et al., 1983). Again, correct orientation was assessed by restriction enzyme analysis.

BAL31/Linker Mutagenesis of p958

p958 was linearized by digestion with Cla I and treated with BAL31 as described previously (Zuniga et al., 1983). The digestion products were ligated to a 25-base pair synthetic oligonucleotide linker (Fig. 1) prepared as described above. Ligation products were transformed into *E. coli* strain MC1061 as described previously (Zuniga et al., 1983). Individual clones were characterized by restriction enzyme analysis, and the deletion end points of selected clones were determined by DNA sequence analysis (Maxam and Gilbert, 1977). Two clones derived from this procedure are E43 and E2 (see text, Fig. 1*A*).

Oligonucleotide-directed Mutagenesis

The Bam HI-Cla I insert of E43 (see above) was cloned into the bacteriophage M13 vector mp10 which was cut with Bam HI and Acc I. Recombinant bacteriophage was grown preparatively[2] and used as substrate for oligonucleotide-directed mutagenesis exactly as described by Newman et al. (1983). The reaction products were analyzed (by agarose gel electrophoresis and Southern blotting [Southern, 1975] with the mutagenic oligonucleotide as a probe) and transformed in *E. coli* JM103. Transformants containing recombinant bacteriophage were plated and resulting plaques were transferred directly to nitrocellulose filters. Filters were prepared for hybridization (Benton and Davis, 1977) and prehybridized at 37°C in 6X SSPE (1× SSPE is 0.18 M NaCl, 1 mM EDTA, 10 mM $NaPO_4$ buffer, pH 7.0), 10× Denhardt's, 50 μg/ml boiled salmon sperm DNA, then hybridized at 37°C for 20 h with 1.5×10^5 cpm/ml ^{32}P-labeled oligonucleotide in 6× SSPE, 5X Denhardt's, 50 μg/ml boiled salmon sperm DNA. Filters were washed in 6× SSPE at increasing temperatures (37, 43, 48, 55, and 60°C) for 3 min each and examined by autoradiography after each wash. Plaques which were only positive at the wash temperature nearest the predicted T_m of the perfect duplex were picked and plaque purified three times. At each purification step the hybridizations were repeated to ensure that only recombinant phage-bearing mutated inserts were selected. The mutant gene fragments were characterized by restriction enzyme analysis (loss and appearance of restriction sites as a consequence of the introduced mutation), and the sequence of the mutants was confirmed by dideoxy sequencing methods.[2] The inserts were retrieved from mp10 by digestion with Bam HI and Hind III and were cloned into pUC8 which was cut with the same enzymes. After introduction of Cla I linkers at the Hind III site of the resulting plasmids (Zuniga et al., 1983), the 3′ half of the gene was regenerated by inserting the Cla I insert of p903 as described above.

Cell Lines, Transformation, Cloning, and Cell Growth

All procedures for growth of mouse L cells and for transformation, selection, cloning, and maintenance of transformants were exactly as described previously (Zuniga et al. 1983). The names and features of all transfected genes are discussed in the text with the exception of p2001, which encodes the signal peptide and first two external domains of H-$2L^d$ (Goodenow et al., 1983). This clone was used as a negative control (Zuniga et al., 1983).

Monoclonal Antibodies (mAb's)

mAb's (30.5.7, 28.14.8 [both anti-H-$2L^d$; Ozato et al., 1980], and 84.17.2) and their purification have been described (Zuniga et al., 1983).

Radioimmunoassay (RIA)

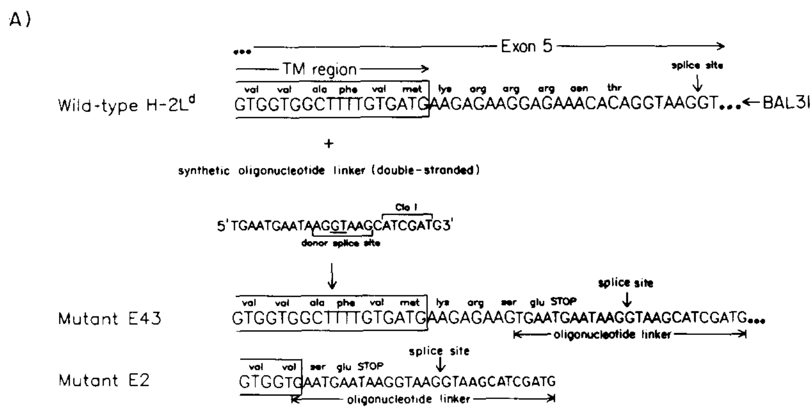
RIA was performed exactly as described (Zuniga et al., 1983), except in experiments for measuring cell surface turnover of H-2 proteins (see below).

Cell Surface Turnover Measurements

Cloned cell lines were plated at 5×10^4 cells/well in flat-bottomed 96-well plates (Corning Glass Works, Corning, NY) the day before RIA. Adherent cells were washed in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supple-

[2] Strauss, E. C., J. A. Kobori, G. Siu, and L. E. Hood, manuscript submitted for publication.

A)



B)

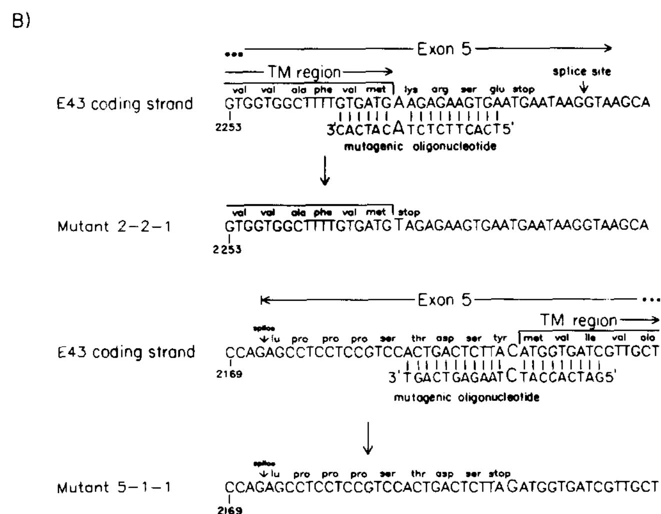


Figure 1. Generation of truncated H-2L^d genes by in vitro mutagenesis. Only the relevant region of exon 5 of the H-2L^d gene is shown. The predicted amino acid sequences (in three-letter code) and termination codons (denoted STOP) are shown above the DNA sequences. TM regions are indicated by boxes and the donor splice sites are indicated by downward arrows. The subclones of the 27.5 H-2L^d gene (Moore et al., 1982) which were used as substrates for mutagenesis and in vitro mutagenesis strategies are described in the text and in Materials and Methods. (*A*) BAL 31/linker strategy. The direction of BAL 31 nuclease digestion is indicated on the right by an arrow. The synthetic oligonucleotide linker DNA (in smaller type than H-2L^d DNA) contains a Cla I restriction site which was used to generate DNA fragments for DNA sequence analysis and for subsequent subcloning procedures. (*B*) Oligonucleotide-directed mutagenesis. The mismatched base of each mutagenic oligonucleotide is indicated in larger type. The numbering of bases is from Moore et al. (1982) as modified in the data bases of Genbank, Bolt, Beranek, and Newman, Inc. (Cambridge, MA).

mented with 10% fetal calf serum (FCS) and then incubated at 4°C for 1 h in 50 μl of mAb (10 μg/ml 30.5.7, anti-H-2L^d or 0.7 μg/ml 84.17.2, anti-H-2K^k) in the same medium. Cells were washed three times in medium, then incubated at 37°C in same for varying periods of time (0, 5, 15, 30, 45, 60, 75, or 90 min). Cells were then washed with ice cold Hank's balanced salt solution containing 0.2% FCS, 0.004% sodium azide, and incubated in 50 μl of the same Hank's solution containing 10^5 dpm ^{125}I-protein A (Amersham Corp., Arlington Heights, IL) at 4°C for 1 h. After four washes in the ice cold Hank's solution, the cells were lysed in 100 μl of 0.5% Triton X-100, 0.05 M Tris-HCl, pH 7.5, and counted. The data were plotted and the slopes of the decay of ^{125}I-protein A bound to the cell surface were measured.

Biosynthetic Labeling of Cells, Immunoprecipitation, and PAGE

Cloned cell lines were plated at 3–4 × 10^6 cells per T75 tissue culture flask (Corning Glass Works) the day before use. For long-term labeling, cells were incubated for 8 h with 100 μCi/ml [^{35}S]methionine (Amersham Corp., 1,345 Ci/mmol) in 2.5 ml methionine-deficient medium (deficient Dulbecco's modified Eagle's medium, Irvine Scientific) supplemented with 5% dialyzed FCS. In pulse-chase experiments, the monolayers were pulsed at 37°C for 15 min with [^{35}S]methionine, washed with complete medium (Zuniga et al., 1983), and then incubated at 37°C for varying periods of time in the complete medium supplemented with 10 mM methionine. For the 0 chase time point, cells were harvested immediately. For labeling of secreted proteins, cells were labeled as above except that FCS was omitted from the medium. Supernatants were concentrated to 300–500 μl in Centricon 10 devices (Amicon Corp., Danvers, MA) prior to immunoprecipitation. Cells were harvested by trypsinization, diluted 10-fold in complete medium supplemented with 10% FCS and 100 μg/ml soybean trypsin inhibitor, and then washed in phosphate-buffered saline. The cell pellets were resuspended at 10^7 cells/ml in lysis buffer (0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min. Lysates were clarified by centrifugation and then made to 1% Triton X-100 and 5 mM EDTA. Immunoprecipitation with mAb and protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was essentially as described by Anderson and Blobel (1983). Immunoprecipitated proteins were analyzed by SDS PAGE (Laemmli, 1970) on 15% polyacrylamide gels followed by fluorography with Autofluor (National Diagnostics, Inc., Somerville, NJ).

Endoglycosidase H Sensitivity Assays

Following immunoprecipitation of pulse-labeled cloned cell lines with 30.5.7 (anti-H-2L^d) and 84.17.2 (anti-H-2K^k) as described above, immunoprecipitated proteins were eluted with 0.1% SDS, 0.01 M Tris-HCl, pH 7.5, 0.01 M dithiothreitol. Aliquots of eluates were incubated overnight at 37°C with 0.1 mU endoglycosidase H (Endo H) (Miles Laboratories Inc., Naperville, IL) in 0.1 M sodium citrate, pH 5.3, 1 mM phenylmethylsulfonyl fluoride, and then run in parallel with untreated samples on SDS polyacrylamide gels (Laemmli, 1970) as described above. Fluorograms were analyzed by quantitative densitometry, and the data for all chase periods were plotted to determine the time required for half the pulse-labeled molecules to become resistant to Endo H cleavage, as determined by altered mobility on the gel.

Results

Construction of Genes Encoding Truncated H-2L^d Proteins

In previous studies (Zuniga et al., 1983), we found that an H-2L^d protein having a cytoplasmic tail of only seven amino

acids (lysine-arginine-arginine-arginine-asparagine-threonine-alanine) rather than the normal 31-amino acid tail (Moore et al., 1982), is expressed at high levels on the surfaces of transfected mouse L cells. The presence of one or more basic amino acids on the cytoplasmic side of the TM segment is a feature which histocompatibility antigens share with other integral membrane proteins (Warren, 1981). We have constructed mutant H-2L^d genes to test the possibility that the basic residues which flank the TM segment (lysine-arginine-arginine-arginine) comprise a required sequence for cell surface expression of H-2 molecules.

Two mutagenesis strategies were used for these studies. In the first, BAL31 nuclease deletion mutagenesis was applied to alter the 3′ end of exon 5, which encodes the TM segment and the adjacent lysine-arginine-asparagine-threonine (amino acids 307–312) sequence (Fig. 1*A* and Materials and Methods). The BAL31 deletion products were then ligated to a synthetic oligonucleotide linker bearing stop codons in every reading frame and a donor splice site (Fig. 1*A*). This strategy should result in an array of mutant H-2L^d genes encoding properly spliced mRNAs whose protein products terminate prematurely. Mutants E43 and E2 were obtained by this approach. Mutants 2-2-1 and 5-1-1 were generated by the introduction of termination codons at specific sites by oligonucleotide-directed mutagenesis of an H-2L^d segment cloned in an M13 bacteriophage vector (Fig. 1*B* and Materials and Methods). All four mutant genes retain the exons encoding the signal sequence and the three external regions (not shown in Fig. 1). Restriction enzyme and DNA sequence analyses of the mutant gene constructs confirmed the structure of the four truncated genes. Mutant E43 has a full-length TM segment and retains two basic residues in its four-amino acid cytoplasmic tail, lysine-arginine-serine-glutamic acid (Fig. 1*A*). Mutant E2 has a TM segment which has lost four hydrophobic amino acids and has a two-amino acid hydrophilic tail, serine-glutamic acid (Fig. 1*A*). These two mutants are useful for determining if charge (in particular, positive charge) is essential for the cell surface expression of H-2L^d protein. Mutant 2-2-1 has an intact TM segment; it terminates with the last methionine of the TM segment (Fig. 1*B*). Hence, this H-2L^d protein can be used to determine whether the hydrophilic tail serves as a membrane anchor, or if it is required for transport of the H-2L^d protein to the cell surface. The fourth mutant used in this study, 5-1-1, lacks both the TM segment and the cytoplasmic tail, but has eight hydrophilic amino acids beyond the end of the β_2-M-binding domain (Fig. 1*B*). Hence, this protein should be secreted into the culture medium by transfected cells, and thus, is a negative control for the E43, E2, and 2-2-1 mutant H-2L^d genes in cell surface expression experiments.

Cell Surface Expression of H-2L^d Molecules That Have Varying Numbers of Basic and/or Hydrophilic Residues at the End of the TM Segment

The mutant H-2L^d genes were introduced with the Herpes Simplex Virus thymidine kinase (tk) gene into tk$^-$ mouse L cells as described previously (Zuniga et al., 1983), and L cell transformants were cloned by limiting dilution. Cloned cell lines were analyzed by cell surface RIA with mAb directed against the foreign H-2L^d and endogenous H-2K^k histocompatibility antigens. Independent clones derived from cultures transfected with any given H-2L^d gene can vary considerably

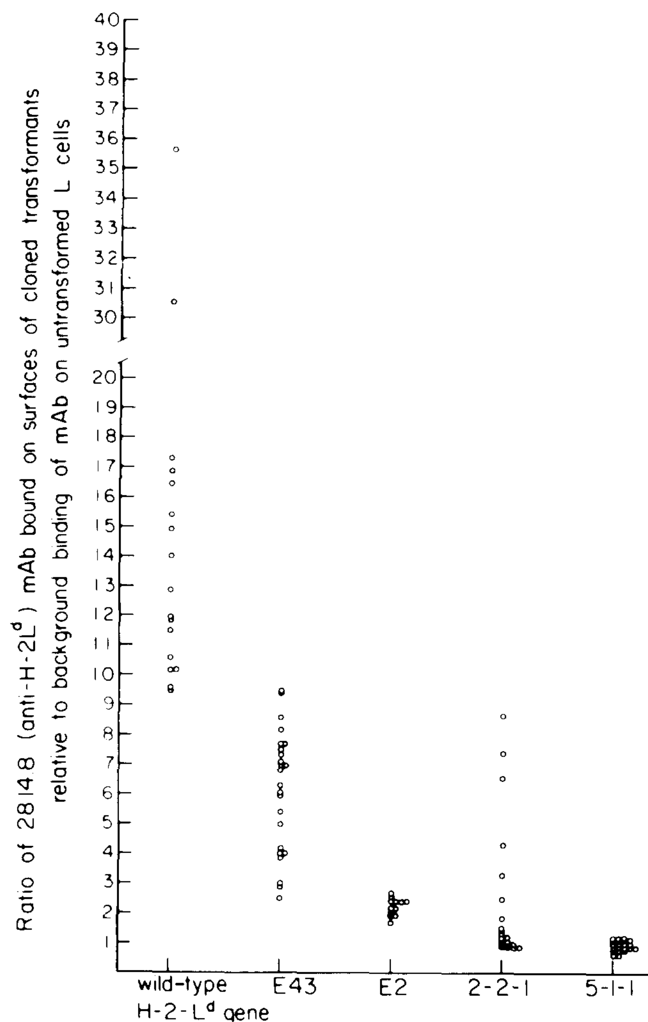


Figure 2. Cytoplasmic tail length and amino acid composition effects on cell surface expression of H-2L^d in L cells. Each circle represents a single measurement of H-2L^d expression on a single clone relative to background binding of mAb on mouse L cells using the 28.14.8 (anti-H-2L^d) mAb in a cell surface RIA (Zuniga et al., 1983). The names of the H-2L^d genes introduced into the L cells are indicated below, and their distinguishing features are discussed in the text. Many of the clones, including those discussed in Tables I–IV, were analyzed multiple times. Nonparametric analyses (Siegel, 1956) of these data show that the pairwise differences between wild-type H-2L^d and all the mutants are statistically significant to a confidence of $P < 0.001$, as are the pairwise differences comparing each of the mutants with each other, except for E43 and 2-2-1.

among each other for the cell surface expression of H-2L^d determinants (Fig. 2). For example, there is an approximately fourfold difference between the highest and lowest expressing clones which were derived from an L cell culture transfected with the full-length H-2L^d gene (Fig. 2). In contrast, the variation in cell surface expression of the H-2K^k antigen is usually no greater than 20–30% (data not shown). Any given clone, however, continuously expresses the same relative level of cell surface H-2L^d and H-2K^k antigens, even after many passages in culture (data not shown). Clonal variation in the level of expression of the protein product of a transfected gene is not uncommon and is presumably due to differences in functional gene copy number (Barbosa et al., 1982), a parameter which cannot be controlled in gene transfection proce-

dures. In spite of this variation, it is clear that the truncated H-2L^d proteins are expressed on the cell surface at lower levels than the full-length molecule. We suggest that this lower level of cell surface expression is due to the disruption of the lys-arg-arg-arg- sequence, because a previously described mutant, BAL911, in which this sequence is intact, is expressed at high levels on the cell surface (Zuniga et al., 1983). The difference in cell surface expression of the BAL911 mutant and the mutants described herein becomes obvious when one calculates the ratio of the average amount of ^{125}I–protein A bound (after previous incubation with 28.14.8 mAb) by transformants which have received a mutant H-2L^d gene relative to the average amount of ^{125}I–protein A bound by transformants which have received the full-length H-2L^d gene. In the case of BAL911 the value is 1.0, whereas in the case of mutants E43, E2, and 5-1-1, the values are 0.40, 0.14, and 0.06, respectively. In the case of 2-2-1 the value for this ratio is 0.33, if only cell surface–positive transformants are included in the calculation, and 0.16 if all transformants are included. The data (not shown) from cytofluorometric analyses of the H-2L^d and the endogenous H-2K^k antigens on the cloned transfectants are consistent with the clonal nature of the cell lines, and confirm the lower cell surface expression of the E43, E2, and 2-2-1 H-2L^d gene products relative to endogenous H-2K^d gene products. As expected, the 5-1-1 mutant H-2L^d protein is not expressed on the cell surface in any of the 34 clones which were transfected with this gene (Fig. 2 and data not shown), because it lacks the TM segment essential for retention in the plasma membrane (Fig. 1 *B*). The 5-1-1 protein can be immunoprecipitated from culture supernatants of cloned transfectants (Fig. 3) and thus is synthesized and secreted. We conclude from these results that the TM segment is necessary and sufficient for cell surface expression of the H-2L^d integral membrane protein and that there is no absolute requirement for a cytoplasmic tail of any particular length or composition, at least not in mouse L cells.

Turnover of Truncated and Wild-type H-2L^d in Mouse L Cells

There are several possible reasons for the lower cell surface expression of these truncated H-2L^d proteins. The most obvious possibility is that the proteins are synthesized in smaller amounts or are unstable once synthesized. Comparable amounts of H-2L^d protein can be immunoprecipitated from transformants expressing wild-type, E43, or 2-2-1 H-2L^d gene products, all of which have full-length TM segments (Table I). Moreover, pulse-chase experiments and immunoprecipitation studies showed that the wild-type, E43, and 2-2-1 H-2L^d proteins have comparable turnover rates (Table II and data not shown). However, relative to the wild-type H-2L^d, a comparably smaller fraction of the immunoprecipitable H-2L^d is expressed on the cell surface in most of the clones transfected with the E43 and 2-2-1 genes (columns 4 and 5 of Table I), suggesting that these mutant proteins are retarded in their transport through the cell or that the mutant proteins are less stable once on the cell surface. In the case of the E2 protein, the low level of expression might be due to an overall lower synthesis (Table I), as well as proteolytic instability (Table II). The low H-2L^d expression in E2 transfectants has hampered further analysis of this mutant protein.

Our failure to detect cell surface H-2L^d antigen in two-thirds of the 2-2-1 transformants (14 out of 21 clones) is not

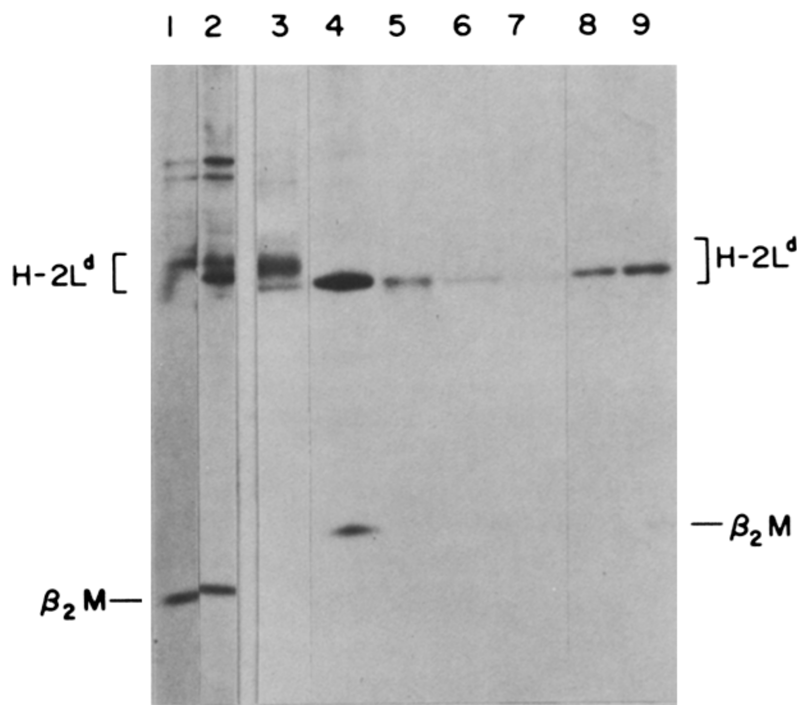


Figure 3. Immunoprecipitation of wild-type and mutant H-2L^d polypeptides from L-cell transformants. Biosynthetic labeling, preparation of cell lysates and culture supernatants, immunoprecipitation, PAGE, and fluorography were performed as described in Materials and Methods. Immunoprecipitates shown in lanes *1* and *2* were formed with mAb 30.5.7 while those in lanes *3–9* were made with mAb 28.14.8. Immunoprecipitates formed with the latter antibody do not always contain quantitative amounts of β_2M, perhaps because the 28.14.8 determinants is on the β_2M-binding domain (Evans et al., 1982). The presence of more than one band in the H-2 region of the gel is presumably due to differential glycosylation, since in pulse-chased material, these bands collapse into one band after treatment with Endo H (Fig. 4 and data not shown). Longer exposures of the fluorogram show that there is β_2M in every immunoprecipitate. Lanes *1–7* show immunoprecipitates from lysates of the following cell lines: lanes *1* and *3*, 27.5.27 D-1 (transformed with the wild-type H-2L^d); lane *2*, C482H11 (transformed with E43 mutant with a four-amino acid cytoplasmic tail). The cell lines analyzed in lanes *4–7* all received the 2-2-1 mutant (tailless H-2L^d); lane *4*, DD1; lane *5*, DD5; lane *6*, DD10; lane *7*, DG3. DD5 and DD10 are cell surface–positive for H-2L^d (see Table 1) whereas DD1 and DG3 are not. Lanes *8–9* show immunoprecipitates from culture supernatants of 5-1-1 transformants BC7 (lane *8*) and BF5 (lane *9*). The aberrantly slow mobility of the 5-1-1 polypeptide is presumably due to the fact that the large hydrophobic domain which has been removed from 5-5-1 contributes very little to the apparent molecular weight of H-2L^d. Similar results have been reported for secreted mutants of influenza hemagglutinin (Gething and Sambrook, 1982) and for VSV G protein (Rose and Bergmann, 1983). Positions of the H-2L^d and β_2M polypeptides are indicated. Lanes *1* and *2* are from a different gel than lanes *3–9*.

Table I. Intracellular and Cell Surface Expression of Wild-type and Mutant H-2L^d Genes in Cloned Cell Lines

Cloned cell line	H-2L^d gene	Cytoplasmic tail	H-2L^d cell surface expression	Total immunoprecipitable H-2L^d
27.5.27 D-1	WT	31-a.a. tail, (4 basic residues)	1.0	1.0
C482H11	E43	4-a.a. tail (2 basic and 1 acidic residue)	0.3	1.0
D262C10	E2	TMΔ 4-a.a., 2-a.a. tail (1 acidic residue)	0.1	0.1
DD5	2-2-1	no cyt. tail	0.5	0.6
DD10	2-2-1	no cyt. tail	0.3	0.7
DD1	2-2-1	no cyt. tail	ND	1.0

Cell surface expression of H-2L^d by transfected L cells was determined by RIA with mAb 28.14.8 as previously described (Zuniga et al., 1983) using the same number of cells for all cell lines. The binding of ^{125}I-protein A by clone DDI was not above the background binding by untransfected L cells. H-2L^d proteins were immunoprecipitated from equal numbers of cells for all cell lines with mAb 28.14.8. Samples were subsequently analyzed by SDS PAGE to confirm that the immunoprecipitated counts were in the form of H-2L^d. The values given are based on scintillation counting (in 10 ml of Hydrofluor [National Diagnostics, Inc., Somerville, NJ]) of [^{35}S]methionine in aliquots of the immunoprecipitates prior to loading on the gel. In columns 4 and 5, the data are normalized relative to the value obtained with the wild-type H-2L^d gene. TM Δ 4 indicates that the TM segment lacks 4 hydrophobic amino acids.
WT, wild-type. *ND*, not detected.

due to the fact that these cells synthesize too little H-2L^d protein to be detected by our methods, nor to the fact that the 2-2-1 mutant protein is not recognized by the antibodies. In fact, comparable amounts of H-2L^d antigen can be immunoprecipitated from cell surface–negative and cell surface–positive transformants (Table I and Fig. 3), even after an 8-h chase (Table II). The retention within the cell of the tailless 2-2-1 H-2L^d protein is not due to a failure of the mutant protein to associate with β_2M (Figs. 3 and 4). The two types of 2-2-1 transformants retained their respective H-2L^d cell surface-positive and -negative phenotypes after passage in culture for several months. Hence this clonal variability for the cell surface expression of this integral membrane protein is stable and clone specific.

This striking clonal difference in the ability of cells to properly transport integral membrane proteins lacking cytoplasmic tails to the cell surface is unexpected. For several reasons, it is highly improbable that this difference is due to a DNA recombination event generating a gene encoding an H-2L^d protein with a cytoplasmic tail in those clones that express 2-2-1 protein on the cell surface. First, the transfection experiment included as controls other H-2L^d genes which either encode secreted H-2L^d (mutant 5-1-1) or an H-2L^d with only the first two external regions (see Materials and Methods) (Zuniga et al., 1983), and in neither case were cell surface expression of H-2L^d antigenic determinants detected. Secondly, the H-2L^d proteins synthesized by 2-2-1 transfectants which express H-2L^d antigen on the cell surface and from clones which express it only intracellularly can be immunoprecipitated with two different mAb's and are indistinguishable on polyacrylamide gels (Fig. 3). Thus, any putative recombination event must have been favored in the 2-2-1 transfection, must have generated a protein with the same apparent molecular weight, and must have affected only the carboxyl-terminal portion of the glycoprotein and done so similarly in all of the seven clones which express the 2-2-1 H-

Table II. Intracellular Stability of Wild-type and Mutant H-2L^d

Cell line	H-2L^d gene	Mutation	Ratio: H-2 immunoprecipitable 8 h chase/0 h chase: H-2L^d 30.5.7	H-2L^d 28.14.8	H-2K^k 84.17.2
27.5.27 D-1	WT	—	0.6	1.7	1.0
27.5.27 FC3	WT	—	0.6	0.5	0.7
C482H11	E43	4-a.a. tail	0.7	0.5–0.8	0.7
D262C10	E2	TM segment Δ4-a.a. and 2-a.a. tail	—	0.3	—
DD5	2-2-1	no cyt. tail	1.5	2.3	—
DD10	2-2-1	no cyt. tail	0.7	0.6	0.6
AE11	2-2-1	no cyt. tail	0.4–0.7	0.4–0.8	0.6
DD1	2-2-1	no cyt. tail	0.4–0.6	0.7–0.8	0.7

H-2L^d proteins were pulse-chase-labeled as described in Materials and Methods. After immunoprecipitation with the mAb indicated, samples were counted and analyzed as described in the legend to Table I. The data are expressed as the ratio of immunoprecipitable cpm [^{35}S]methionine present after an 8-h chase with 10 mM methionine relative to the value obtained with no chase. TM Δ 4-a.a. indicates that the TM segment lacks four hydrophobic amino acids. 2-2-1 transfectants DD5 and DD10 are cell surface-positive for H-2L^d, whereas AE11 and DD1 are not.
WT, wild-type.

2L^d on the cell surface.

Another possible explanation for these results is that the clonal variation observed is unrelated to sorting of tailless integral membrane proteins, but rather reflects the preferential use of alternative splice sites in some of the cloned cell lines, resulting in H-2L^d proteins with unexpected carboxyl termini. The considerable homology among class I genes makes S_1 nuclease analysis extremely difficult. Although we have no data ruling out the possibility that the clonal variation among 2-2-1 transformants is due to use of alternative splice sites, we consider it to be unlikely. To minimize the possible use of cryptic splice sites, we took care to reconstruct the original donor sequence in the synthetic linker used to generate mutants E43 and E2 (Fig. 1 *A*). Mutant E43 is the parent of mutants 2-2-1 and 5-1-1 and differs from these two by a single base pair removed from the donor splice site of exon 5 by 18 and 91 bp, respectively (Fig. 1 *B*). Thus, we would have expected that if alternative splicing is occurring in 2-2-1 precursor mRNAs that it should also be occurring in the E43 RNAs. Admittedly, alteration of the carboxyl terminal of the E43 protein might have gone undetected by our analyses (see below). However, others have shown that extensive deletions or alterations within exon and intron regions flanking splice sites do not affect splicing fidelity (Khoury et al., 1979; Gruss and Khoury, 1980; Wieringa et al., 1983).

Cell Surface Stability of Wild-type and Truncated H-2L^d Proteins

If the basic residues serve as a membrane anchor sequence, perhaps by interacting with the negatively charged phospholipid groups on the inner side of the membrane (Ploegh et al., 1981; Mescher, 1982), then the lower cell surface expression might be due to a higher turnover rate of molecules that reside on the plasma membrane. Turnover measurements of cell surface H-2L^d and H-2K^k proteins were made by incubating adherent cells at 4°C with mAb against either the H-2L^d or the H-2K^k protein (see Materials and Methods), washing, then incubating at 37°C for varying periods of time, and then

measuring the amount of ^{125}I–protein A that binds to the cell surface. For most of the cell lines the H-2K^k protein turns over more rapidly than the H-2L^d protein. This result is not surprising, since different H-2 molecules are internalized (Leserman et al., 1985) and shed (Emerson et al., 1980) at different rates. To compensate for clonal differences in H-2 cell surface expression, the data in Table III are expressed as the cell surface turnover of the exogenous H-2L^d (wild-type or mutant) protein relative to the endogenous H-2K^k protein. These measurements reveal no significant systematic differences in the turnover rate among wild-type H-2L^d, H-2L^d molecules having two basic residues in a four-amino acid cytoplasmic tail (mutant E43), and H-2L^d molecules having no cytoplasmic tail at all (mutant 2-2-1) (Table III). Moreover, we have not been able to detect significant quantities of H-2L^d in the culture supernatants of these transformants, under conditions that permit detection of the secreted 5-1-1 H-2L^d protein. These results argue against the notion that the cluster of basic residues on the cytoplasmic side of the TM segment is an anchoring moiety essential for stability of these membrane proteins.

The Basic Residues and Glycosylation of H-2L^d Proteins

Like many other glycoproteins (Rothman and Lodish, 1977), H-2 molecules acquire oligosaccharides (rich in mannose residues) at asparagine residues during translation on the ER (Dobberstein et al., 1979) and undergo further processing of oligosaccharides and attachment of complex sugars in the Golgi apparatus (Rothman, 1981). Endo H cleaves mannose-rich oligosaccharides, but not complex oligosaccharides. To test the possibility that a slower rate of processing is responsible for the lower cell surface expression of mutant E43 and 2-2-1 proteins, we assessed the sensitivity of H-2L^d proteins to Endo H. Cells were pulsed with [^{35}S]methionine, incubated with an excess of unlabeled methionine for varying periods of time, and processed for immunoprecipitation (see Materials and Methods). Aliquots of immunoprecipitated H-2 antigens were incubated with Endo H, and digestion products were analyzed by PAGE. This method was also used to determine if the clones that express only intracellular 2-2-1 H-2L^d antigen do so because of a failure to transport this mutant protein from the ER to the Golgi apparatus. As an internal control for each cell line, the rate of glycosylation of the endogenous H-2K^k was also measured.

For most of the cell lines examined, the H-2K^k antigen is processed much more rapidly than is the H-2L^d protein (mutant or wild-type) (Table IV). The slow processing rate for H-2K^k protein in the 27.5.27 D-1 cell line is interesting, since the cell surface expression of H-2K^k antigen on this clone is low relative to the H-2L^d antigen (Table IV, column 4) and relative to H-2K^k antigen on other clones (data not shown). A difference in maturation rate between H-2D^d and H-2K^d molecules in SL2 lymphoma cells has also been observed (Dobberstein et al., 1979), so the fact that the exogenous H-2L^d protein is processed more slowly than the endogenous H-2K^d antigen is unlikely to be related to the fact that it is the product of an introduced gene. It might be related to the fact that the H-2L^d molecule has three glycosylation sites (Hansen et al., 1983), whereas the H-2K^k molecule has only two (Arnold et al., 1984). However, there is no evidence that the number of carbohydrates affect transport through the Golgi apparatus. Alternatively, it is possible that H-2K^k associates with β_2M more efficiently than H-2L^d, resulting in a more efficient processing of H-2K^k (Krangel et al., 1979).

A comparison of cloned cell lines expressing the different H-2L^d proteins on their cell surface reveals that the lower cell surface expression of the E43 and 2-2-1 H-2L^d proteins, relative to the wild-type, correlates with a slower rate of glycosylation (Table IV). Surprisingly, in cloned cell lines which express the 2-2-1 H-2L^d protein intracellularly and which fail to express it on the cell surface, the tailless H-2L^d molecule can be glycosylated rapidly (Table IV and Fig. 4). In clone DD1, for example, all of the immunoprecipitable H-2L^d protein is resistant to Endo H cleavage after a 30-min chase (Fig. 4*A*), in contrast to DD10 which expresses H-2L^d on the cell surface (Fig. 4*B*).

Table III. Length of Cytoplasmic Tail Has No Effect on the Stability of H-2L^d on the Plasma Membrane

Cell line	H-2L^d gene	Length of cyt. tail	Ratio turnover rates H-2L^d/H-2K^k
27.5.27 D-1	WT	31	1.0 (2)
27.5.27 5F6	WT	31	0.5
27.5.27 C-1	WT	31	0.5
27.5.27 4B5	WT	31	0.4
C482H11	E43	4	0.4 (2)
DD5	2-2-1	0	1.0 (2)
DD10	2-2-1	0	0.3
AA2	2-2-1	0	0.5

Cell surface turnover of H-2L^d and H-2K^k glycoproteins was determined as described in Materials and Methods. The data are expressed as the turnover rate of H-2L^d relative to the turnover rate of H-2K^k in the same experiment. Numbers in parentheses indicate the number of measurements made. Values varied no more than 10%.

WT, wild-type.

Discussion

The basis of these studies is the previous observation that H-2L^d proteins having truncated intracellular tails are transported to the cell surface where they are expressed in a functional form (Zuniga et al., 1983; Murre et al., 1984). Since these truncated proteins retain four basic residues adjacent to the TM segment, in the current studies we sought to determine whether these basic amino acids are required for the expression of high levels of H-2L^d protein on the cell surface. We constructed genes encoding mutant H-2L^d proteins which lack most of the cytoplasmic tail and have sustained further deletions in the best amino acid cluster (Fig. 1, *A* and *B*).

An underlying assumption of this type of approach to structure–function studies is that observed changes in the level of expression and/or cellular localization of the protein product of a modified gene are the consequence of the introduced alterations in protein structure. However, it is possible that in addition there is variation among cells in the ability to sort and transport certain kinds of proteins. This is a nontrivial concern when dealing with cell lines maintained in culture for a long period of time. To address this possibility we cloned the transfectants and used only cloned cell lines for the analysis of the expression and cellular localization of the altered H-2L^d gene products. The cells were grown under conditions which maintain the selective pressure for the co-

Table IV. Rate of Acquisition of Endo H Resistance by H-2 Molecules

Cloned cell line	Introduced H-2L^d	Cytoplasmic tail	H-2L^d/H-2K^k expression on cell surface	$t_{½}$ (in min) Endo H resistance by H-2 molecule	
				H-2L^d (exogenous)	H-2K^k (endogenous)
27.5.27 FC3	WT	31-a.a., 4 basic	1.0	50	18
27.5.27 D-1	WT	31-a.a., 4 basic	4.0	69	69
C482H11	E43	4-a.a., 2 basic, 1 acidic	0.4	132	18
DD10	2-2-1	no cyt. tail	0.53	171	<30
DD1	2-2-1	no cyt. tail	ND	<15	<15
AE11	2-2-1	no cyt. tail	ND	<15	—

Immunoprecipitation of H-2L^d (with mAb 30.5.7) and H-2K^k (with mAb 84.17.2) from pulse-chase-labeled cells, Endo H digestions, polyacrylamide gel analysis, and quantitative densitometry, and data analysis were performed as described in Materials and Methods. The chase periods with 10 mM methionine were 0, 2, 4, 6 and 8 h, except in the case of DD1 for which the chase periods were 0, 15 min, 30 min, 1 h, 2 h, and 4 h. The amount of Endo H-sensitive material at t_0 was generally >70%. The data in columns 5 and 6 are expressed as the time (in minutes) required for half the pulse-labeled molecules to become resistant to Endo H cleavage. The data in column 4 are derived from the RIA analysis shown in Fig. 2 and from RIAs that were performed within a few days before cells were set up for Endo H experiments.

WT, wild-type.

ND, not detected.

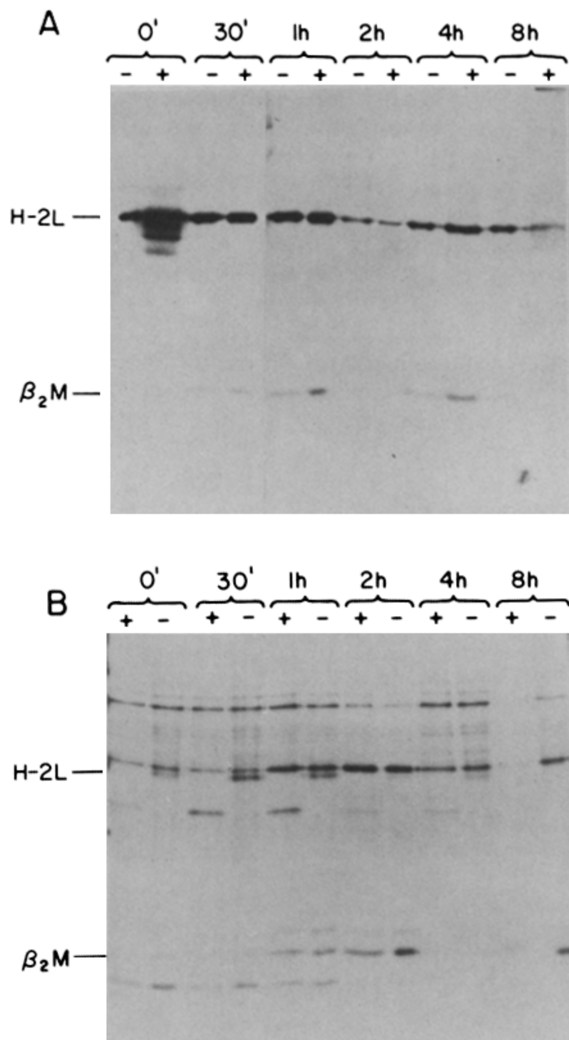


Figure 4. Processing of tailless H-2L^d polypeptides in clones which do and do not express them on the cell surface. Immunoprecipitation of H-2L^d (with mAb 30.5.7) from pulse-chase-labeled cells, Endo H digestions, PAGE, and fluorography were performed as described in Materials and Methods and in Table IV. Endo H treatment (+) or lack thereof (−), as well as length of chase period, are indicated above each lane, whereas the positions of H-2L^d and β_2M are indicated on the left. The cloned cell lines expressing the tailless H-2L^d are as follows: (*A*) DD1 (cell surface negative for H-2L^d) and (*B*) DD10 (cell surface positive for H-2L^d). The data for DD10 in Table IV were not derived from this fluorogram. Longer exposure of the fluorograms reveals H-2L^d and β_2M in every lane.

transformed tk gene (Zuniga et al., 1983) so as to minimize the loss of introduced DNA sequences.

The Intracellular Tail Is Not a Membrane Anchor

Our results show that the basic amino acids on the cytoplasmic side of the TM region enhance the high level of expression on the cell surface of the H-2L^d protein (Fig. 2), but are not absolutely required. An H-2L^d protein having a cytoplasmic tail of four amino acids, two of which are basic (E43), an H-2L^d molecule with an acidic two-amino acid intracellular tail (E2), and an H-2L^d protein with no cytoplasmic tail (2-2-1) can each be expressed on the cell surface. However, the levels of these proteins are lower than that of another truncated H-2L^d protein with a seven-amino acid tail which includes four basic residues (Zuniga et al., 1983; data not shown). Cell surface turnover measurements on cloned transfectants show that the hydrophobic TM region itself is sufficient for the stable membrane integration of the H-2L^d protein (Table III). The basic residues on the intracellular tail may have functions unrelated to tethering the integral membrane protein on the plasma membrane. These functions also are not related to the ability of the H-2L^d molecule to interact with the cytotoxic T cell receptor, since clones expressing the E43, E2, or 2-2-1 H-2L^d molecules are effectively killed in cytotoxicity assays (Forman, J., and M. Zuniga, manuscript in preparation). We have observed, however, that the tailless 2-2-1 molecule differs from the wild-type and other mutant H-2L^d glycoproteins in its lateral mobility on the cell surface and in capping (Edidin, M., and M. Zuniga, unpublished data).

Intracellular Transport of Truncated H-2L^d Glycoproteins

The intracellular stability of the E43 and 2-2-1 mutant H-2L^d proteins is indistinguishable from that of H-2L^d protein with a full-length cytoplasmic tail, or that of the endogenous H-2K^k molecule (Table II). However, in clones which express these truncated glycoproteins on the cell surface, they are transported to the *trans*-Golgi relatively slowly (Table IV). In

contrast, in clones in which the tailless 2-2-1 protein is expressed only intracellularly, this truncated molecule can be processed through the Golgi apparatus very rapidly (Table IV). These results suggest that the introduced mutations cause a perturbation in the intracellular transport of the H-2L^d protein. It is interesting to note that in other studies in which integral membrane proteins were converted into secretory proteins, these mutant proteins were glycosylated more slowly than their nonmutant counterparts (Rose and Bergman, 1983; Sveda et al., 1982; Florkiewicz et al., 1983). If intracellular transport is regulated by the selective binding of specific receptors in the ER to nascent glycoproteins (Strous and Lodish, 1980; Fitting and Kabat, 1982; Ledford and Davis, 1983), then the conversion of an integral membrane protein to a secretory protein might result in different compartmentalization. Similarly, the rapid maturation of the 2-2-1 H-2L^d gene product in clones that express it intracellularly might reflect a different compartmentalization of this novel glycoprotein in these clones. However, in clones expressing cell surface H-2L^d the differences in rate of glycosylation do not correlate with a change in the final destination of the molecule. The altered processing rates of the 2-2-1 protein in these clones and of the E43 protein bring to mind the various mutant VSV G proteins, Rous sarcoma virus *env* glycoproteins, and influenza hemagglutinins with altered cytoplasmic tails which acquire Endo H resistance more slowly than their full-length homologues (Rose and Bergmann, 1983; Wills et al., 1984; Doyle et al., 1985). In other studies with H-2L^d antigen (Zuniga et al., 1983; Murre et al., 1984) and Semliki Forest virus E2 protein (Garoff et al., 1983), rates of transport were not examined. However, alteration or truncation of the cytoplasmic tail did not diminish cell surface expression of these proteins. Taken together, the results from studies on several integral membrane proteins suggest that both primary amino acid sequence and conformation are important for protein sorting and transport.

Clonal Variation for the Transport of Tailless H-2L^d to the Cell Surface

A remarkable result of these studies is that cloned transfectants which synthesize an H-2L^d protein with a normal TM region but no cytoplasmic tail vary in a clone-specific fashion with regard to cell surface expression of this molecule. This striking variation was not observed with the other mutant H-2L^d proteins. It is possible that clonal variation does occur among cloned transfectants which received the E43 and E2 gene constructs, but that the variation affects the level of cell surface expression to a lesser degree and not in an all-or-none fashion as in the case of the 2-2-1 gene product. Recent cDNA and genomic sequence evidence showing that Thy-1 antigen is an integral membrane protein with no cytoplasmic tail (Seki et al., 1985) is consistent with the observation that in some fibroblast clones a tailless H-2L^d can be transported to the cell surface. Since Thy-1 is limited in its tissue distribution (Williams and Gagnon, 1982), it is interesting to consider the possibility that the sorting pathway by which tailless integral membrane proteins are transported to the cell surface is used in some cell types but not in others. The fibroblast clones which express tailless H-2L^d on the cell surface may have deviated in their sorting mechanisms during the long-term culture of the parent line. Immunofluorescence microscopy and subcellular fractionation studies should make it possible to localize the H-2L^d protein in those clones which accumulate the 2-2-1 protein intracellularly. Since the transfected clones are stable, it will also be interesting to see whether the clones which do and do not express the tailless H-2L^d protein on their surfaces can transport an introduced Thy-1 gene product to the plasma membrane. Thus, the clones characterized in the present study should be useful for further analyses of intracellular transport pathways used by integral membrane proteins in mammalian cells.

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