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Alternative processing of H-2D^d pre-mRNAs results in membrane expression of differentially phosphorylated protein products

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Two distinct mRNA species encoding the mouse major histocompatibility antigen H-2D^d have been identified in BALB/c spleen cells as well as in cultured cell lines expressing this cell surface glycoprotein. The alternate transcripts of H-2D^d arise from either removal or inclusion of exon VII (encoding I_2) during pre-mRNA processing. The relative levels of each kind of H-2D^d transcript varied considerably between different cell types, and in all cells examined both forms of alloantigen were expressed on the cell membrane. Antigen derived from both types of transcript reacted with H-2D^d-specific monoclonal antibodies, whereas only protein lacking the 13 amino acids of I_2 reacted with a specific antiserum raised against a predicted exon VI/VIII fusion peptide. Those H-2D^d proteins translated from full length, but not smaller, transcripts were phosphorylated in resting and phorbol myristate acetate-stimulated BALB/c spleen cells, suggesting that the major site of *in vivo* phosphorylation is within the highly conserved sequence encoded by exon VII. Thus alternative splicing of pre-mRNA transcripts is a mechanism which leads to membrane expression of two forms of H-2D^d, one of which lacks a major site of phosphorylation.

Key words: H-2 class I antigens/alternative splicing/membrane expression/anti-peptide antibodies/*in vivo* phosphorylation

Introduction

Class I major histocompatibility antigens are ubiquitous cell surface molecules which provide membrane targets for recognition by effector T cells during allograft rejection and virus infection (Klein, 1975; Snell *et al.*, 1976). These molecules have three distinct extracellular domains (known as N, C1, and C2), a hydrophobic transmembrane region (TM) and an intracellular sequence. The striking polymorphism of class I genes is mainly attributable to the extensive diversity in the external N and C1 domains which are thought to interact with the antigen-specific receptor on T cells. The structure of the C2 (α3) domain and the class I light chain, β_2-microglobulin, contribute little to the polymorphism of class I antigens (Steinmetz and Hood, 1983). However, there is considerable structural variation within the 30–40 amino acid intracytoplasmic region of class I antigens (Moore *et al.*, 1982; Weiss *et al.*, 1983; Kvist *et al.*, 1983; Arnold *et al.*, 1984; Sher *et al.*, 1985). This region is encoded by 3′ sequences of exon V (TM), and by exons VI, VII and VIII (I_1, I_2 and I_3). Apart from primary sequence differences within this region, alternative pre-mRNA processing has been suspected as another source of the observed structural variation (Lew *et al.*, 1986a). Alternate splicing of these exons has been deduced from analysis of class I cDNA clones derived from both SWR and DBA/2 mouse liver (Lalanne *et al.*, 1983, 1985; Kress *et al.*, 1983). In addition, two of the four cDNAs derived from the H-2D^d gene of SV40-transformed BALB3T3 cells were found to represent transcripts in which exon VII was missing such that exon VI and VIII were contiguous (Brickell *et al.*, 1983, 1985).

Despite these studies there has been no direct evidence for membrane expression of proteins corresponding to any of the alternatively spliced class I transcripts described above. A recent study of surface expressed products derived from a truncated H-2D^d gene (a genetically engineered mutant lacking exons II and III) revealed two discrete membrane-bound proteins differing in mol. wt by 1500 daltons (McCluskey *et al.*, 1986). These results suggested that two forms of the H-2D^d molecule may be expessed normally.

Here we report the mRNA and protein expression of H-2D^d in normal, virally transformed and tumor cells expressing this antigen as well as L cells transfected with chimeric or deleted genes derived from H-2D^d. We show that in all cells examined, H-2D^d is expressed as two distinct mRNAs which arise from the alternative removal or inclusion of exon VII during pre-mRNA processing. These alternate transcripts give rise to two membrane forms of this antigen, the smaller of which lacks 13 amino acids encoded by exon VII (I_2). The smaller H-2D^d molecules differ functionally from those which possess I_2 in that they fail to undergo constitutive or phorbol myristate acetate (PMA)-induced phosphorylation *in vivo*.

Results

Alternative splicing of H-2D^d-derived pre-mRNAs

To identify H-2D^d mRNA transcripts representative of the normal splicing pattern (containing sequences derived from exons I–VIII inclusively) or of the alternative splicing pattern represented by the H-2D^d cDNA pAG64 (lacking the sequences of exon VII), we performed ribonuclease protection studies on a panel of cells expressing normal H-2D^d, chimeric or deleted forms of H-2D^d and appropriate negative controls. Cytoplasmic or total cellular RNA was hybridized with an excess of a 324 nucleotide (nt) ^{32}P-labelled antisense RNA probe derived from a subclone of an H-2D^d cDNA lacking exon VII (Figure 1A). Duplex hybrids of RNA molecules in which exon VII sequences were present would be expected to yield a 279 nt fragment and a smaller, 11 nt, fragment (unlikely to be detected) following ribonuclease digestion. Hybrids formed with transcripts lacking exon VII would generate a 290 nt nuclease-resistant fragment. Apart from residual undigested probe, no major protected fragments were identified in any of the RNAs derived from cells not expressing wild-type or transfected forms of H-2D^d: JT1.1, BW5147 and DMT9.10.2 (Figure 1B). By contrast, both the 290 and 279 nt fragments were protected by RNAs derived from cells expressing either wild-type or genetically manipulated genes containing 3′ sequences of H-2D^d: DMT9.9.4, JT6.4.1, M12.4.1, BALB/c spleen cells, SV3T3, BALB3T3, T4.8.3 and DMT33.32.1 (Figure 1B). The ratio of the two protected fragments varied between 0.05 and 0.4 in different cells and the

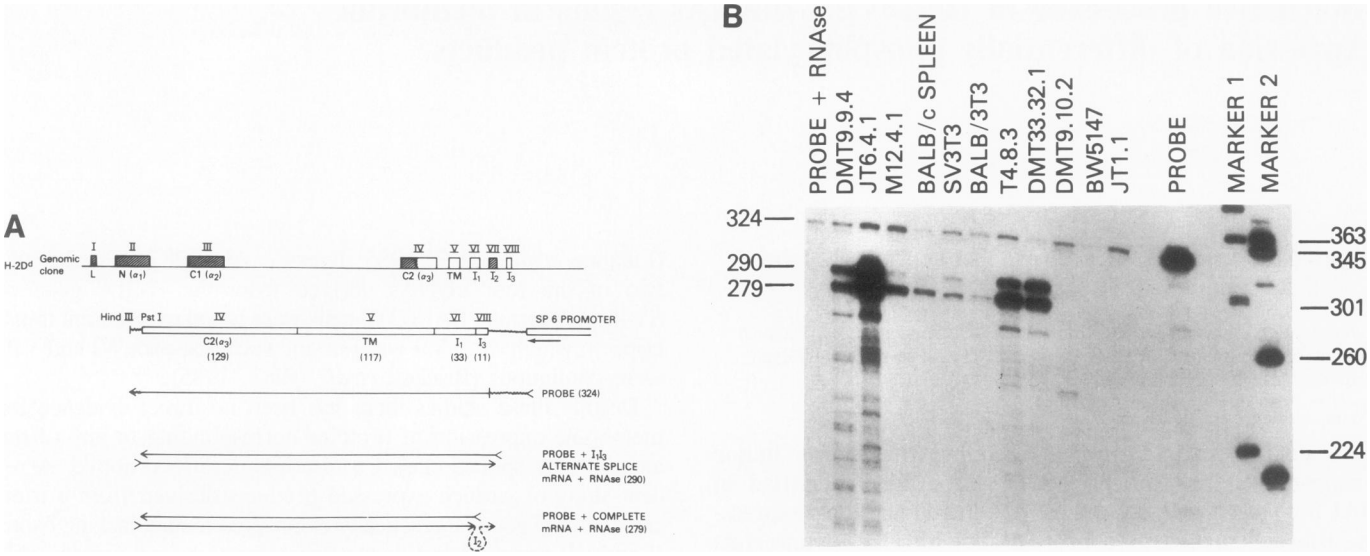

Fig. 1. Ribonuclease protection analysis of H-2D^d mRNA transcripts in normal and cultured cells. **(A)** Schematic representation of the gene structure of H-2D^d, the probe and expected protected fragments. Genomic sequences present in the probe are shown as open boxes. The expected protected fragments of this probe are schematically indicated. **(B)** RNA from the indicated cell lines or tissues was hybridized to the probe described in (A), and treated with a mixture of RNase A and T1. Protected RNA hybrids were fractionated by electrophoresis in a 6% polyacrylamide denaturing sequencing gel. RNAs are derived from DMT9.9.4, an L cell transformed with an exon II/III deletion of H-2D^d with an I-A$^k_\beta$-derived promoter (McCluskey *et al.*, 1986); JT6.4.1, an L cell transformed with a class II/class I recombinant gene previously described (McCluskey *et al.*, 1985); M12.4.1, a BALB/c B cell lymphoma (Kim *et al.*, 1983); SV3T3, SV40-transformed BALB3T3 cells; BALB3T3; T4.8.3, a H-2D^d-transformed L cell (Margulies *et al.*, 1983); DMT33.32.1, an L cell transformed with an exon II/III deletion of H-2D^d (McCluskey *et al.*, 1986); DMT9.10.2, an L cell transformed with an exon II/III deletion of H-2L^d (McCluskey *et al.*, 1986); BW5147, an AKR T cell lymphoma; and JT1.1, a tk$^-$ L cell transformant (McCluskey *et al.*, 1985). Minor protected bands were present in some of the high expressor L cell RNAs upon longer exposure of autoradiographs. These bands may represent other splicing events in these cells. In particular, a 246-nt protected fragment seen in several experiments may represent an exon V/VIII spliced transcript.

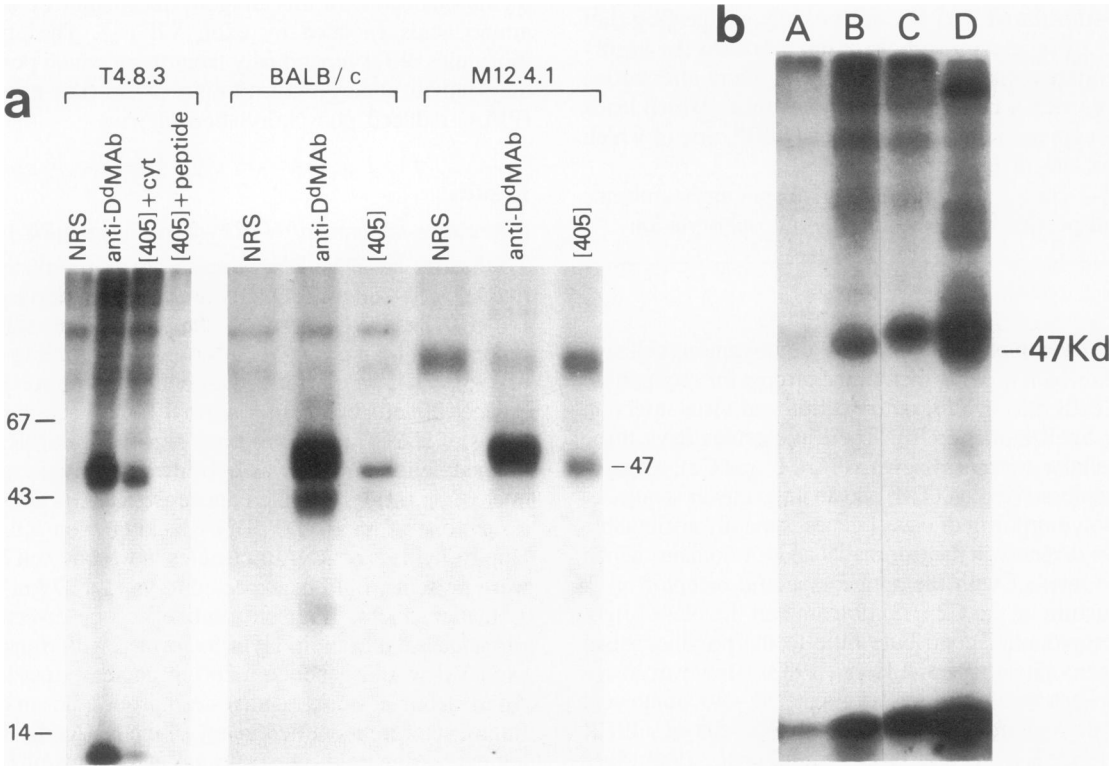

Fig. 2. Immunoprecipitated surface-labelled H-2D^d proteins of normal and cultured cells. Cells were surface-labelled by lactoperoxidase-catalyzed iodination, detergent extracts were prepared and the post-nuclear supernatants were used for immunoprecipitation as described in the Materials and methods. Autoradiograph of a 12% gel. (**a** left panel): NRS, normal rabbit serum; anti-H-2D^d Mabs, 34.2.12 (anti-H-2D^d C2 domain) and 34.5.8 (anti-H-2D^d N/C1 domain); [405] + cyt, antiserum [405] mixed with 20 μg of a soluble peptide fragment of cytochrome c; [405] + peptide, antiserum [405] mixed with 20 μg of the H-2D^d exon VI-VIII fusion peptide KGGDYALAPV. (Right panel): autoradiograph of a 10% gel. Abbreviations as above except that antiserum [405] was not mixed with synthetic peptide. **(b)** Autoradiograph of a 10% gel. Labelled lysate of T4.8.3 was reacted with antiserum [405] after first preclearing with anti-H-2D^d Mabs **(lane A)** or with NRS **(lane B)**. Conversely, lysate was reacted with anti-H-2D^d Mabs after first preclearing with antiserum [405] **(lane C)** or with NRS **(lane D)**.

smaller fragment (indicative of the larger transcript) was always predominant. These results were confirmed in simultaneous experiments using a uniformly labelled antisense RNA probe derived from an H-2D^d cDNA encoding exons IV, V, VI, VII and VIII (unpublished data). Proportionately more of the smaller transcripts were present in L cell transfectants compared with the B lymphoma M12.4.1, BALB3T3 cells or BALB/c spleen cells. Moreover, the L cells expressing deleted genes (DMT9.9.4; DMT33.32.1) or deleted and shuffled genes (JT6.4.1) expressed nearly equivalent amounts of the two mRNA forms.

Alternative H-2D^d transcripts are expressed as different membrane-bound forms of antigen

To determine whether cells expressing alternatively spliced H-2D^d mRNAs also expressed the corresponding membrane-bound protein we synthesized an oligopeptide, KGGDYALAPV, the sequence of which represents the fusion of residues encoded by exon VI and VIII of H-2D^d. This was used to raise a rabbit anti-peptide antiserum [405] that specifically precipitates only molecules containing the fusion sequence. The specificity of antiserum [405] for these residues has previously been demonstrated (McCluskey *et al.*, 1986). Attempts to generate an antibody against the exon VI, VII, VIII-encoded peptide have been unsuccessful. Cells were surface ^{125}I-labelled and their detergent lysates were reacted with either normal rabbit serum, rabbit antiserum [405] or anti-H-2D^d monoclonal antibodies (Mabs) that recognize epitopes on the extracellular C2 domain (Mab 34.2.12) or epitopes on the N/C1 domains (Mab 34.5.8) (Figure 2A, B). Thus membrane-bound H-2D^d antigen with or without exon VII residues would be expected to immunoprecipitate with anti-H-2D^d Mabs; however, only those molecules lacking exon VII residues should immunoprecipitate when reacted with rabbit antiserum [405]. The anti-H-2D^d Mabs immunoprecipitated a heterogeneous glycoprotein of ~47–50 kd from T4.8.3 (Figure 2A, left panel), BALB/c spleen cells and M12.4.1 (Figure 2A, right panel). In addition a heterogeneous band of mol. wt 40–44 kd was variably precipitated from BALB/c spleen cell extracts. From these same cells, antiserum [405], which recognizes specifically the carboxy-terminal exon VI/VIII-encoded fusion peptide, precipitated a discrete protein of 47 kd, which co-migrated with the leading edge of H-2D^d antigen immunoprecipitated with anti-H-2D^d Mabs. The reactivity of antiserum [405] was specifically inhibited by a synthetic oligopeptide representing residues derived from the fusion of exons VI and VIII, but was not inhibited by an equivalent amount of cytochrome C peptide (Figure 2A, left panel). This further verifies the specificity of antiserum [405] for H-2D^d antigen lacking the residues encoded within exon VII as previously described (McCluskey *et al.*, 1986).

The preclearance of radiolabeled T4.8.3 cell lysate with anti-H-2D^d Mabs removes >90% of the antigen precipitated by antiserum [405] (Figure 2B, lanes A and B). Conversely, exhaustive preclearance of cell lysate using antiserum [405] only removes the smaller molecular weight component of the H-2D^d antigen precipitated by anti-H-2D^d Mabs, whereas the higher molecular weight forms of this antigen are not removed by antiserum [405] (Figure 2B, lanes C and D). Partial endoglycosidase F digestion of immunoprecipitates from labelled BALB/c spleen-detergent lysates indicated that the antigens precipitated with antiserum [405] have two major N-linked carbohydrate additions as predicted (unpublished data). Thus the protein precipitated by antiserum [405] (Figure 2) represents H-2D^d molecules translated from mRNAs in which exon VII sequences have been eliminated, while that precipitated by anti-H-2D^d Mabs represents all those

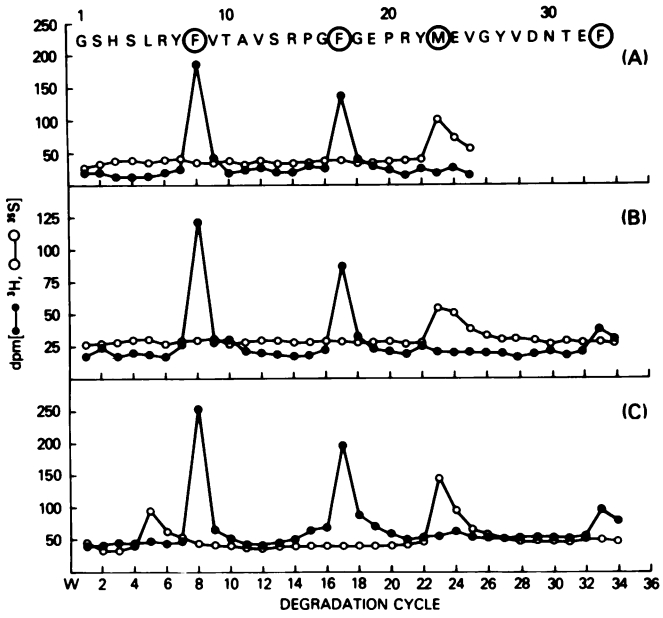

Fig. 3. Amino-terminal radiosequence analysis of antiserum [405] and monoclonal antibody 34.2.12-precipitated proteins. BALB/c spleen cells were labelled with [^{3}H]phenylalanine and [^{35}S]methionine. Detergent lysates were immunoprecipitated and fractionated by electrophoresis on 10% polyacrylamide gels containing SDS. The bands obtained by immunoprecipitation with Mab 34.2.12 and antiserum [405] were recovered by electroelution and subjected to automated sequence determination. **(A)** Upper portion of the labelled band precipitated by Mab 34.2.12, **(B)** lower portion of the labelled band precipitated by Mab 34.2.12, and **(C)** single band precipitated by antiserum [405]. Indicated above, in the single letter amino acid code, is the sequence of H-2D^d as described (Sher *et al.*, 1985). In **(C)** an ^{35}S peak at cycle 5 represents <5% of the sequenced antigen calculated relative to the methionine at position 23. Since H-2L^d, but not H-2D^d, has a methionine at position 5 this suggests that antiserum [405] precipitates small quantities of H-2L^d.

molecules which possess serological epitopes present on the extracellular part of H-2D^d.

The two forms of H-2D^d surface antigen have identical amino-terminal radiosequence

To demonstrate that the antigen precipitated with antiserum [405] was unequivocally H-2D^d, BALB/c spleen cells were radiolabelled with [^{35}S]methionine and [^{3}H]phenylalanine. The specifically immunoprecipitated bands were identified, purified, and subjected to amino-terminal radiosequence analysis (Figure 3). Amino-terminal radiosequence analysis of the upper (Figure 3A) and lower (Figure 3B) portions of the heterogeneous 47–50 kd band precipitated by anti-H-2D^d Mabs and the single band identified by antiserum [405] (Figure 3C) demonstrated methionine at position 23 and phenylalanine at positions 8, 17 and 33. Since a phenylalanine residue at position 17 particularly distinguishes H-2D^d from all other known murine class I antigens (Kimball and Coligan, 1983) these data verify that the antigen recognized by antiserum [405] is H-2D^d. Thus H-2D^d pre-mRNAs are alternatively spliced leading to membrane expression of two forms of the H-2D^d antigen, one of which lacks intracytoplasmic sequences encoded by exon VII. Scrutiny of the predicted amino acid sequences of cloned murine, human, porcine and rabbit class I antigens has revealed the conservation of the sequence Ser-Asp/Glu-X-Ser-Leu within exon VII (Table I). In the case of the human class I molecules, phosphorylation of the second serine of this cluster (serine 335) has been demonstrated unequivocally for HLA-A2 and HLA-B7 (Pober *et al.*, 1978; Guild and Strominger, 1984). The remarkable preservation of this sequence,

Table I. Carboxy-terminal sequences of class I MHC antigens

	Exon V	Exon VI	Exon VII	Exon VIII
Mouse				
		320	330 340	
		|	| |	
H-2D^d	R R R N T	G G K G G D Y A L A P	G S Q S S D M S L P D C K	V
pAG64	- - - - -	- - - - - - - - - - - -	( )	-
H-2L^d	- - - - -	- - - - - - - - - - - -	- - - - - E - - - R - - -	A
H-2D^b	- - - - -	- - - - - - - - - - - -	- - - - - E - - - R - - -	A
H-2K^b	- - - - -	- - - - - - - - - - - -	- - - T - - L - - - - - -	V M V H D P H S L A
H-2K^d	M - - - -	- - - - V N - - - - -	- - - T - - L - - - - G -	V M V H D P H S L A
H-2K^k	- - - - -	- - - - - - - - - - - -	- - - T - - L - - - - - -	V M V H D P H S L A
pH8	- - - - -	- - - - - - - - - - - -	- - - T - - L - - - - - -	V M V H D P H S L A
pH13	- - - - -	- - - - - - - - - - - -	- - - T - - L - - - - - -	A
pH12	- - - - -	- - - - - - - - - - - -	- - - - - E - - - R - - -	A
p5	- - - - -	- - - - - - - - - - - -	- - - T - - L - - - - - -	V M V H D P H S L A
Q-7^d	- - X - -	- - Q - - - C - P - -	- - X - - - R - - - - G -	V M V H D P H S L A
Other species				
H-2D^d	R R R N T	G G K G G D Y A L A P	G S ( ) Q S S D M S L P D C K	V
HLA-A2	- - K S S	D R - - - S - S Q - A	S - D S A - G - - V - - T A - -	-
HLA-A3	- - K S S	D R - - - S - T Q - A	S - D S A - G - - V - - T A - -	-
HLA-AW24	- - N S S	D R - - - S - S Q - A	S - D S A - G - - V - - T A - -	-
HLA-B7	- - K S S	- - - - - S - S Q - A	C - D S A - G - - V - - T A	
pHLA12.4	- K K S S	D R - - - S - S Q - A	S - N S A - G - - V - - T A	
HLA-CW3	- - K S S	- - - - - S C S Q - A	S - N S A - G - - E - - I A - -	A
PD1	- K T R S	- E - - - S - T Q - A	- - D S D - G - - V - - T K D P R	-
PD14	- K K R S	- E - - - S - T Q - A	- - D S A - G - - V - - T K D P R	-
R19-1	K K H S S	D - - - - R - T P - A	- G H R D - G - - D - - M P	

Protein sequences, or the predicted protein sequences are shown for the indicated MHC genes. Exon boundaries have been assigned based upon the amino acid codon derived from a majority of the nucleotides of the codon. References for the listed mouse sequences are: H-2D^d (Sher *et al.*, 1985), pAG64 (Brickell *et al.*, 1983), H-2L^d (Moore *et al.*, 1982), H-2D^b (Reyes *et al.*, 1982; Maloy and Coligan, 1982), H-2K^b (Weiss, 1983), H-2K^d (Kvist *et al.*, 1983), H-2K^k (Arnold *et al.*, 1984), pH8 (presumably H-2K^q), pH13, (presumably H-2K^q), pH12 (q haplotype) (Kress *et al.*, 1983), p5 (H-2K^q-like gene from H-2^{w28}) (Morita *et al.*, 1985), Q-7^d (the reassignment of 27.1) (Steinmetz *et al.*, 1981); for the indicated human sequences: HLA-A2 (Koller and Orr, 1985), HLA-A3 (Strachan *et al.*, 1985), HLA-AW24 (N'Guyen *et al.*, 1985), HLA-B7 (Sood *et al.*, 1985), pHLA12.4 (Malissen *et al.*, 1982), HLA-CW3 (Sodoyer *et al.*, 1984); for the swine sequences PD1 and PD14 (Satz *et al.*, 1985); and for the rabbit sequence R19 (Marche *et al.*, 1985). Homology of the mouse sequences with those of human, pig and rabbit is maximized by introducing a 3-amino acid residue gap in murine Exon VII-encoded sequences as suggested (Guild and Strominger, 1984). In addition, the recent publication of *Tla* gene and cDNA sequences should be noted (Obata *et al.*, 1985; Fisher *et al.*, 1985; Chen *et al.*, 1985). Their carboxy-terminal protein sequences do not preserve the structure Ser-Asp/Glu-X-Ser-Leu, but do contain Ser-Leu in this region.

despite considerable variation in the neighboring amino acid residues, argues that phosphorylation within this region plays an important functional role. Hence, the elimination of these conserved residues from the class I molecule might have a significant bearing on subsequent function.

Differential in vivo phosphorylation of the two forms of H-2D^d antigen

To determine whether murine class I antigens are phosphorylated *in vivo*, BALB/c spleen cells were labelled with [^{32}P]orthophosphate, lysed, and reacted either with anti-H-2D^d Mabs or antiserum [405], and the immunoprecipitates were analyzed by SDS – polyacrylamide gel electrophoresis and autoradiography (Figure 4). A single band of ^{32}P-labelled H-2D^d antigen was precipitated by anti-H-2D^d Mabs from normal spleen cell lysates. However, no specific signal was detected when antiserum [405] was used. Analysis of BALB3T3 cells, T4.8.3, and DMT9.9.4 demonstrated the *in vivo* phosphorylation of the higher molecular weight form of H-2D^d but not the lower molecular weight form specifically precipitated by antiserum [405] (unpublished data). This result also indicated that, although several additional amino acid residues encoded by exons V and VI can serve as an *in vitro* substrate for phosphorylation (Guild and Strominger, 1984), the only sites used in the *in vivo* intracellular phosphorylation of H-2D^d depend upon the structure encoded by exon VII.

To determine whether intracellular phosphorylation of H-2D^d was responsive to regulation, [^{32}P]orthophosphate-labelled BALB/c spleen cells were stimulated for 30 min with PMA for comparison with unstimulated cells (Figure 4). A two- to three-fold induction of ^{32}P-labelled H-2D^d antigen was observed following this treatment. However, even with PMA induction, no phosphorylation of the lower molecular weight forms of H-2D^d was detected. Analysis of [^{32}P]orthophosphate-labelled DBA/2 B cells has verified these results (unpublished data). Furthermore, the proliferative stimulation of resting DBA/2 B cells by culture supernatants containing B cell stimulatory factor-1 and anti-μ antiserum leads to augmented phosphorylation of many of the same phosphoproteins induced by PMA, but does not increase phosphorylation of H-2D^d (unpublished data). Thus phosphorylation of H-2D^d does not arise non-specifically in response to any stimulus inducing protein phosphorylation of membrane-bound antigens.

Discussion

Alternate processing of H-2D^d pre-mRNAs clearly leads to membrane expression of two forms of this antigen, only one of which is phosphorylated *in vivo*. Despite the evidence for alternative splicing at both the 5′ and 3′ ends of other class I mRNAs, the observation discussed here is among the first examples verifying protein expression derived from differentially processed RNA transcripts. Recently, alternate transcripts encoding two distinct

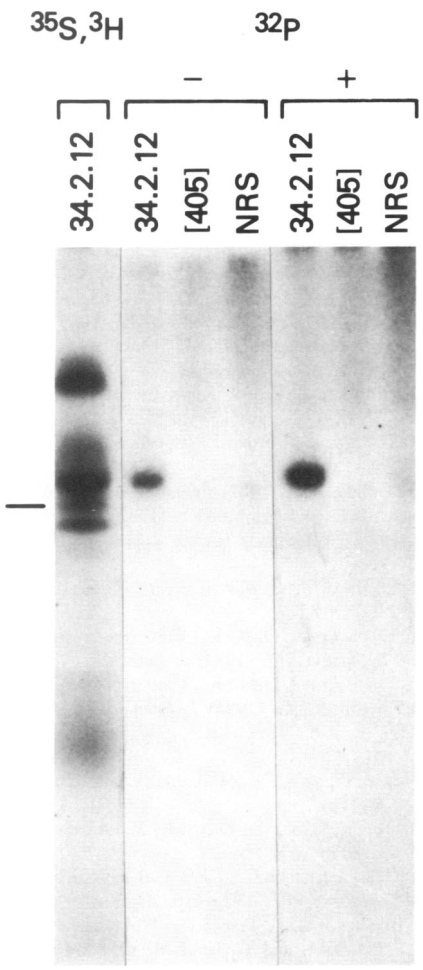

Fig. 4. Resting spleen cell and PMA-induced [^{32}P]orthophosphate-labelled H-2D^d proteins. BALB/c spleen cells were labelled with [^{32}P]orthophosphate as described in the Materials and methods and then treated for 30 min with either PMA (+ **lanes**) in ethanol at 10 ng/ml final concentration or an equal amount of ethanol alone (unstimulated; − **lanes**). Detergent lysates were reacted in the presence of phosphatase inhibitors with normal rabbit serum (NRS), rabbit antiserum [405] or monoclonal antibody 34.2.12, and processed for electrophoresis and autoradiography. For comparison, an aliquot of the [^{3}H]phenylalanine [^{35}S]methionine-labelled spleen cell immunoprecipitate was run in an adjacent lane on the same polyacrylamide gel with a mol. wt of 47 kd indicated by a line. The precise nature of the ^{3}H, ^{35}S-labelled proteins migrating with a mol. wt of 43 kd is unknown.

forms of exon VIII of the H-2K^b molecule have been demonstrated (Lew *et al.*, 1986b). It has been suggested that some of the observed differences in intracytoplasmic sequences expressed by H-2D versus H-2K molecules, and between certain H-2K alleles, are probably accounted for by polymorphism within the intron between exons VII and VIII (Lew *et al.*, 1986b; Archibald *et al.*, 1986). This polymorphism subsequently affects the position of lariat branch point formation (Padgett *et al.*, 1984; Ruskin *et al.*, 1984) which alters the choice of the acceptor splice junction, leading to variation in the mature mRNA and its translated protein products. Such structural variation within the intracytoplasmic sequences of class I antigens may lead to distinct pathways of intracellular processing, compartmentalization, sorting, transport and recycling of these molecules. Analysis of L cell clones transfected with H-2L^d mutants lacking any intracytoplasmic residues demonstrated striking clonal variation in the membrane expression of these antigens (Zuniga and Hood, 1986). Whereas some clones expressed cell surface membrane-associated antigen, others failed to transport these mutant proteins from the Golgi apparatus to the cell membrane successfully, suggesting a role for class I intracytoplasmic sequences in surface expression. Deletion mutants of H-2L^d lacking most, but not all, intracytoplasmic residues have been shown to be normally recognized by allo- and influenza-specific cytotoxic T lymphocytes (Zuniga *et al.*, 1983; Murre *et al.*, 1984). The less efficient recognition of truncated H-2L^d molecules by vesicular stomatitis virus-specific cytotoxic T lymphocytes may result from undefined cytoskeletal interactions of the cytoplasmic domains of class I molecules (Murre *et al.*, 1984).

Since phosphorylation of the cytoplasmic domains of many growth factor receptors occurs upon ligand − receptor interaction (Cohen *et al.*, 1980; Heldin *et al.*, 1983; Jacobs *et al.*, 1983; Ulrich *et al.*, 1985; Ebina *et al.*, 1985), the phosphorylation of class I molecules may indicate a similar role for their receptor-signalling function. The apparent physical interaction of MHC class I antigens and insulin receptors on mouse liver membranes may implicate H-2 proteins in the transduction of insulin receptor-mediated signalling (Fehlmann *et al.*, 1985). In addition, phosphorylation-dependent signalling by class I antigens may be important during thymic education of developing T lymphocytes, in the generation of class I restricted T-helper function or in interactions of class I molecules in specific tissues during development.

The splicing of eukaryotic pre-mRNAs is extremely accurate, however, diversity of gene expression arising from alternative splicing has been described for IgM (Early *et al.*, 1980), αA-crystallin (King and Piatogorsky, 1983), rat γ-fibrinogen (Crabtree and Kant, 1982), myelin basic protein (Takahashi *et al.*, 1985; deFerra *et al.*, 1985) and Lyt-2 (Zamoyska *et al.*, 1985). Active mechanisms controlling regulation of alternative splicing are critical in adenovirus gene expression (Nevins and Wilson, 1981) and determine the germline specificity of *Drosophila* P element transposition (Laski *et al.*, 1986). In addition the muscle protein troponin T undergoes alternate splicing of pre-mRNAs which appears to be differentially regulated and probably accounts for the observed diversity of its protein isoforms in different cell types (Breitbart *et al.*, 1985). Thus control of expression of the two forms of H-2D^d through differential pre-mRNA processing may constitute a subtle mechanism for modifying class I antigen function. Analogous molecular events may be involved in the expression of other surface structures responsible for intercellular signalling within the immune system, such as class II molecules and T cell receptors.

Materials and methods

Ribonuclease protection analysis

A 290-bp *Pst*I/*Pvu*II fragment spanning most of exon IV (C2 or α3) as well as exons V (TM), VI (I_1) and VIII (I_3), was purified from the H-2D^d cDNA pAG64 (kindly provided by P.J.W.Rigby) (Brickell *et al.*, 1983, 1985; Margulies *et al.*, 1985) and directionally cloned into pGEM2 (Promega Biotech). The vector was digested with *Pst*I and *Sma*I, dephosphorylated with calf intestinal phosphatase and then ligated to the insert using standard protocols (Maniatis *et al.*, 1982). The *Hin*dIII linearized form of this subclone was then used as the substrate for the synthesis of a uniformly ^{32}P-labelled RNA probe using SP6 polymerase (Melton *et al.*, 1984). Cytoplasmic (Mushinski *et al.*, 1980) or total cellular RNA (Chirgwin *et al.*, 1979) was co-precipitated with the probe, redissolved and allowed to anneal overnight at 45°C. Unprotected RNAs were digested with a mixture of RNase A and RNase T1 for 1 h at 37°C followed by treatment with SDS and proteinase K. The sample was solubilized in 95% formamide and subjected to electrophoresis in a 6% polyacrylamide denaturing gel.

Surface iodination and metabolic labelling of cells

Cells were surface-labelled with ^{125}I-carrier-free iodide (Amersham) by lactoperoxidase catalyzed iodination (Haustein, 1975). For metabolic labelling cells were

incubated for 8 h in RPMI deficient in phenylalanine and methionine and supplemented with 2% FCS [^{3}H]phenylalanine (0.5 mCi/ml) and [^{35}S]methionine (0.5 mCi/ml). For ~^{32}P]orthophosphate labelling, BALB/c spleen cells, at 1.6 × 10^{7}/ml, were incubated for 2 h in RPMI deficient in phosphate and supplemented with 10% dialyzed FCS and [^{32}P]orthophosphate (Amersham) at 1 mCi/ml. Cells were washed three times in chilled PBS supplemented with a cocktail of phosphatase inhibitors containing 4 mM EDTA, 100 mM pyrophosphate (Sigma), 100 mM sodium fluoride (Sigma) and 4 mM sodium vanadate (Aldrich or Fisher). Detergent lysates were separated from nuclei by a 20-min centrifugation in an Eppendorf centrifuge. Extracts were precleared with affinity-purified rabbit anti-mouse Ig (Cappel, Cooper Biomedical) and reacted with protein A – Sepharose (Pharmacia). Specific immune precipitations were carried out in the same way except that the protein A – Sepharose beads were washed, and bound material was solubilized in 2% SDS, 0.15 M 2-mercaptoethanol followed by electrophoresis in 10 or 12.5% polyacrylamide gels containing SDS (Maizel, 1971). Gels were autoradiographed as described (Laskey and Mills, 1975).

Amino terminal radiosequence analysis and phosphorylation studies

Following radiolabelling and polyacrylamide gel electrophoresis, the bands obtained by immune precipitation with Mab 34.2.12 (anti-C2, H-2D^{d}) and rabbit antiserum [405] were recovered by electroelution and subjected to automated sequence determination in a sequenator as described (Coligan *et al.*, 1983).

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References

Archibald,A.L., Thompson,N.A. and Kvist,S. (1986) *EMBO J.*, **5**, 957–965.
Arnold,B., Burget,H.-G., Archibald,A.L. and Kvist,S. (1984) *Nucleic Acids Res.*, **12**, 9473–9486.
Breitbart,R.E., Nguygen,H.T., Medford,R.M., Destree,A.T., Mahdavi,V. and Nadal-Ginard,B. (1985) *Cell*, **41**, 67–82.
Brickell,P.M., Latchman,D.S., Murphy,D., Willison,K. and Rigby,P.W.J. (1983) *Nature*, **306**, 756–760.
Brickell,P.M., Latchman,D.S., Murphy,D., Willison,K. and Rigby,P.W.J. (1985) *Nature*, **316**, 162–163.
Chen,T.-T., Obata,Y., Stockert,E. and Old,L.J. (1985) *J. Exp. Med.*, **162**, 1134–1138.
Chirgwin,J.M., Prsybyla,G., MacDonald,P.J. and Rutter,W.J. (1979) *Biochemistry*, **18**, 5294–5299.
Cohen,S., Carpenter,G. and King,L.E.,Jr. (1980) *J. Biol. Chem.*, **225**, 4834–4842.
Coligan,J.E., Gates,F.T.,III, Kimball,E.S. and Maloy,W.L. (1983) In Colowick,S.P. and Kaplan,N.O. (eds), *Methods in Enzymology*. Academic Press, New York, pp. 413–432.
Crabtree,G.R. and Kant,J.A. (1982) *Cell*, **31**, 159–166.
deFerra,F., Engh,H., Hudson,L., Kamholz,J., Puckett,C., Molineaux,S. and Lazzarini,R.A. (1985) *Cell*, **43**, 721–727.
Early,P., Rogers,J., Davis,M., Calame,K., Bond,M., Wall,R. and Hood,L. (1980) *Cell*, **20**, 313–320.
Ebina,Y., Ellis,L., Jarnagin,K., Edery,M., Graf,L., Clauser,E., Ou,J.-H., Masiarz,F., Kan,Y.W., Goldfine,I.D., Roth,R.A. and Rutter,W.J. (1985) *Cell*, **40**, 747–758.
Fehlmann,M., Peyron,J.-F., Samson,M., Obberghen,E.V., Brandenburg,D. and Brossette,N. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8634–8637.
Fisher,D.A., Hunt,S.W.,III and Hood,L. (1985) *J. Exp. Med.*, **162**, 528–545.
Guild,B.C. and Strominger,J. (1984) *J. Biol. Chem.*, **259**, 9235–9240.
Haustein,D. (1975) *J. Immunol. Meth.*, **7**, 25–38.
Heldin,C.-H., Ek,B. and Ronnstand,L. (1983) *J. Biol. Chem.*, **258**, 10054–10061.
Jacobs,S., Kull,F.C.,Jr, Earp,H.S., Svoboda,M.E., VanWyck,J.J. and Cuatrecasas,P. (1983) *J. Biol. Chem.*, **258**, 9581–9584.
Kim,J.K., Kanallopoulous-Langevin,C., Mermin,R.M., Sachs,D.H. and Asofsky,R. (1983) *J. Immunol.*, **122**, 549–556.
Kimball,E. and Coligan,J.E. (1983) *Contemp. Top. Mol. Immunol.*, **9**, 1–63.
King,C.R. and Piatagorsky,J. (1983) *Cell*, **32**, 707–712.
Klein,J. (1975) *Biology of the Mouse Histocompatibility Complex*. Springer-Verlag, Berlin.
Koller,B.H. and Orr,H.T. (1985) *J. Immunol.*, **134**, 2727–2733.
Kress,M., Glaros,D., Khoury,G. and Jay,G. (1983) *Nature*, **306**, 602–604.
Kvist,S., Roberts,L. and Dobberstein,B. (1983) *EMBO J.*, **2**, 245–254.
Lalanne,J.-L., Cochet,M., Kummer,A.-M., Gachelin,G. and Kourilsky,P. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7561–7565.
Lalanne,J.-L., Transy,C., Guerin,S., Darche,S., Menlein,P. and Kourilsky,P. (1985) *Cell*, **41**, 469–478.
Laski,F.A., Rio,D.C. and Rubin,G. (1986) *Cell*, **44**, 7–19.
Laskey,R.A. and Mills,A.D. (1975) *Eur. J. Biochem.*, **56**, 335–341.
Lew,A.M., Lillehoj,E.P., Cowan,E.P., Maloy,W.L., van Schravendijk,M.R. and Coligan,J.E. (1986a) *Immunology*, **57**, 3–18.
Lew,A.M., Margulies,D.H., Maloy,W.L., Lillehoj,E.P., McCluskey,J. and Coligan,J.E. (1986b) *Proc. Natl. Acad. Sci. USA*, **83**, 6084–6088.
McCluskey,J., Germain,R.N. and Margulies,D.H. (1985) *Cell*, **40**, 247–257.
McCluskey,J., Bluestone,J.A., Coligan,J.E., Maloy,W.L. and Margulies,D.H. (1986) *J. Immunol.*, **136**, 1472–1481.
Maizel,J.G. (1971) In Maramorosch,K., Koprowski,H. (eds), *Methods of Virology*. Academic Press, New York, pp. 179–246.
Malissen,M., Malissen,B. and Jordan,B.R. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 893–897.
Maloy,W.L. and Coligan,JE.. (1982) *Immunogenetics*, **16**, 11–20.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Marche,P.N., Tykocinski,M.L., Max,E.E. and Kindt,T.J. (1985) *Immunogenetics*, **21**, 71–82.
Margulies,D.H., Evans,G.A., Ozato,K., Camerini-Otero,D., Tanaka,K., Appella,E. and Seidmann,J.G. (1983) *J. Immunol.*, **130**, 463–470.
Margulies,D.H., Evans,G.A., Ozato,K., Camerini-Otero,D., Tanaka,K., Appella,E. and Seidman,J.G. (1985) *J. Immunol.*, **135**, 1537.
Melton,D.A., Krieg,P. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Moore,K.W., Sher,B.T., Sun,T.K., Eakle,K.A. and Hood,L. (1982) *Science*, **215**, 679–682.
Morita,T., Delarbre,C., Kress,M., Kourilsky,P. and Gachelin,G. (1985) *Immunogenetics*, **21**, 367–383.
Murre,C., Reiss,C.S., Bernabeu,C., Chen,L.B., Burakoff,S.J. and Seidman,J.G. (1984) *Nature*, **307**, 432–436.
Mushinski,J.F., Blattner,F.R., Owens,J.D., Finkelman,F.D., Kessler,S.W., Fitzmaurice,L., Potter,M. and Tucker,P.W. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7405–7409.
Nevins,J.R. and Wilson,M.C. (1981) *Nature*, **290**, 113–118.
N'guyen,C., Sodoyer,R., Trucy,J., Strachan,T. and Jordan,B.R. (1985) *Immunogenetics*, **21**, 479–489.
Obata,Y., Chen,Y.-T., Stockert,E. and Old,L.J. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5475–5479.
Padgett,R.A., Konarska,M.M., Grabowski,P.J., Hardy,S.F. and Sharp,P.A. (1984) *Science*, **255**, 898–903.
Pober,J.S., Guild,B.C. and Strominger,J.L. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 6002–6006.
Reyes,A.A., Schold,M. and Wallace,R.B. (1982) *Immunogenetics*, **16**, 1–9.
Ruskin,B., Krainer,A.F., Maniatis,T. and Green,M.R. (1984) *Cell*, **38**, 317–331.
Satz,L.M., Wang,L.-C., Singer,D.S. and Rudikoff,S. (1985) *J. Immunol.*, **135**, 2167–2175.
Sher,B.T., Nairn,R., Coligan,J.E. and Hood,L.E. (1985) *Proc. Natl. Acad. Sci USA*, **82**, 1175–1179.
Snell,G.D., Dausset,J. and Nathenson,S. (1976) *Histocompatibility*. Academic Press, New York.
Sodoyer,R., Damotte,M., Delovitch,T.L., Trucy,J., Jordan,B.R. and Strachan,T. (1984) *EMBO J.*, **3**, 879–885.
Sood,A.K., Pan,J., Biro,P.A., Pereira,D., Srivastava,R., Reddy,V.B., Duceman,B.W. and Weissman,S.M. (1985) *Immunogenetics*, **22**, 101–121.
Steinmetz,M., Moore,K.W., Frelinger,J.E., Sher,B.T., Shen,F.-W., Boyse,E.A. and Hood,l. (1981) *Cell*, **25**, 683–692.
Steinmetz,M. and Hood,L. (1983) *Science*, **222**, 727–733.
Strachan,T., Sodoyer,R., Damotte,M. and Jordan,B.R. (1985) *EMBO J.*, **4**, 887–894.
Takahashi,N., Roach,A., Teplow,D.P., Prusiner,S.B. and Hood,L. (1985) *Cell*, **42**, 139–148.
Ulrich,A., Bell,J.R., Chen,E.Y., Herrera,R., Petruzzelli,L.M., Dull,T.J., Gray,A., Coussens,L., Liao.Y.—C., Tsubokowa,M., Mason,A., Seeburg,

P.H., Grunfeld,C., Rosen,O.M. and Ramachandran,J. (1985) *Nature*, **313**, 756–761.
Weiss,E.H., Golden,L., Zakut,R., Mellor,A., Fahrner,K., Kvist,J. and Flavell,R.A. (1983) *EMBO J.*, **2**, 453–462.
Zamoyska,R., Vollmer,A.C., Sizer,K.C., Liaw,W.C. and Parnes,J.R. (1985) *Cell*, **43**, 153–163.
Zuniga,M.C., Malissen,B., McMillan,M., Brayton,P.R., Clark,S.S., Forman,J. and Hood,L. (1983) *Cell*, **34**, 535–544.
Zuniga,M.C. and Hood,L. (1986) *J. Cell. Biol.*, **102**, 1–10.

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