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Biochemical and Functional Analyses of a Secreted H-2L^d Molecule

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A truncated H-2L^d gene was constructed by deleting the transmembrane and cytoplasmic exons. The truncated H-2L^d gene was introduced into mouse L cells using the thymidine kinase gene as a selectable marker. Transformants were isolated and screened for the presence of truncated H-2L^d antigen. The truncated H-2L^d gene product was present in both the cytoplasm and culture medium, but not on the cell surface. The truncated H-2L^d antigen was stable in culture medium for at least 9 h and was secreted into the medium at a rate similar to the kinetics with which complete H-2 antigens reach the cell surface. Transformants expressing the truncated H-2L^d molecule were not recognized by cytotoxic T lymphocytes specific for the H-2L^d antigen.

The major histocompatibility complex of the mouse consists of a number of genes encoding cell surface proteins which are involved in cellular recognition and control of the immune response (14, 31). A subset of these genes is the class I genes, whose products serve as targets for cytotoxic T cells in allogeneic immune responses such as *in vivo* graft rejection. However, the physiological role of the class I proteins has been shown to be that of restriction elements in T-cell lysis of virus-infected cells (9). The class I molecule consists of two subunits, H-2 (44,000 daltons) and β 2-microglobulin (12,000 daltons), which are noncovalently associated. The H-2 subunit is composed of three extracellular domains, a transmembrane domain, and a cytoplasmic domain (6). Each of the extracellular domains is encoded by separate exons (10, 21, 23). The portions of the H-2 molecule that are involved in the specific interaction between T lymphocytes and target cells, by permitting the specific recognition, remain uncertain. One approach to studying this interaction has been to analyze the ability of T cells to recognize target cells bearing spontaneous, genetically altered H-2 molecules (15). These studies have been extended by the use of recombinant DNA technology to generate altered H-2 genes. H-2 genes altered *in vitro* have been introduced into L cells and studied for their ability to serve as targets for cytotoxic T lymphocytes (CTLs) (1, 2, 11, 24-27, 33).

We have now constructed an H-2 gene that directs the synthesis of a secreted H-2L^d molecule. The biochemical and functional characteristics of this novel H-2 antigen are presented.

Previous work has shown that removal of the transmembrane and cytoplasmic domains of the influenza hemagglutinin and vesicular stomatitis virus G protein results in conversion of the native transmembrane protein into a truncated protein that is secreted into the medium (29, 32).

To obtain a secreted H-2L^d antigen, we decided to take a similar approach. An H-2L^d gene encoding a secreted H-2 antigen was constructed by using restriction enzymes and BAL 31 exonuclease to delete the transmembrane and cytoplasmic domains, as shown in Fig. 1. One constraint imposed on restructuring the gene appears to be that a poly(A) addition site must be present at the 3' end of the gene. Therefore, a DNA segment derived from the IA β gene encoding a poly(A) addition site, three amino acids, and a termination codon (5) was added to the truncated TH-2 gene, resulting in plasmid H-2SEC (Fig. 1). To determine whether the novel recombinant gene is expressed when introduced into mouse L cells, DNA from this gene together with DNA from the herpesvirus thymidine kinase gene was used to cotransform thymidine kinase-negative mouse L cells (12). Twenty transformants were isolated. Northern blot analysis on isolated mRNA with the 3' end of the IA β gene as a probe revealed that five transformants expressed the truncated gene (C. Murre and J. G. Seidman, unpublished results).

To determine that splicing of the ligated IA β exon in the truncated H-2L^d mRNA is similar to that of the IA β mRNA normally expressed in A20, a B-cell lymphoma, S1 analysis of the mRNAs was performed. This analysis was done with a single-stranded, uniformly labeled DNA probe of high specific activity that was prepared by primer extension of a recombinant phage M13 template containing a *Bam*HI-*Eco*RI fragment derived from the murine IA β gene (25; Fig. 2). After the mRNAs were hybridized with the labeled DNA probe, the reaction mixture was digested with S1 nuclease, and the products were analyzed on denaturing polyacrylamide gels (Fig. 2). Only probes that had been hybridized either to mRNA isolated from the transformed L cells (TSEC) or to mRNA isolated from B cells expressing the IA β gene were protected from S1 nuclease digestion. A 90-nucleotide fragment was protected by mRNA isolated from transformant TSEC (Fig. 2, lane C). The protected fragment was identical to the fragment protected by mRNA

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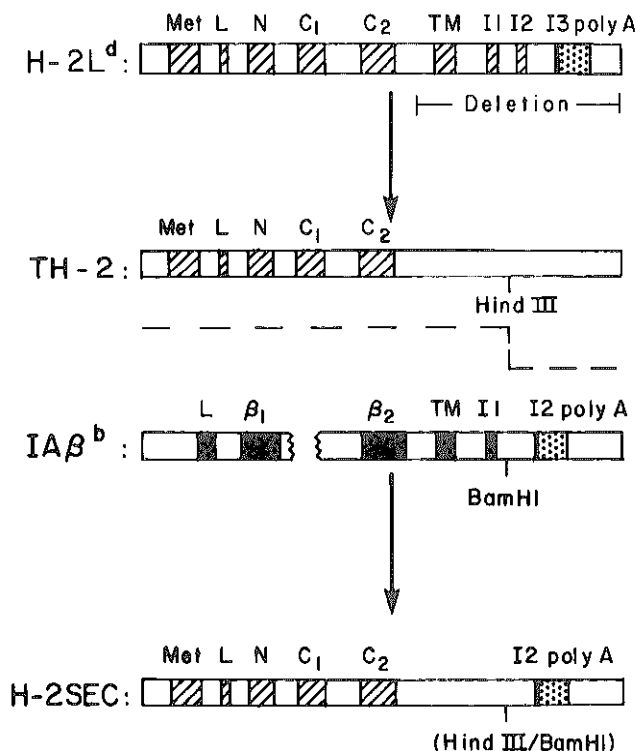


FIG. 1. Construction of an H-2 gene encoding a secreted antigen. A truncated H-2L^d gene was constructed by standard DNA technology (20). The transmembrane and cytoplasmic domains of the H-2L^d gene in plasmid pLL in which the normal H-2 promoter has been replaced by the metallothionein promoter (24) were deleted by using exonuclease BAL 31. The chewing was started from a *Bgl*II site present in the intron between the transmembrane and first cytoplasmic domain (25). The DNA fragments treated with BAL 31 were next ligated to an excess of *Hind*III linker. The exact sites of deletions were determined by restriction mapping and dideoxy sequence analysis (30). One clone had been deleted into the intron between C₂ and TM and was subcloned into plasmid pKC7 (20), resulting in plasmid pTH-2. A 2.5-kilobase *Bam*HI fragment encoding the second cytoplasmic domain, including a termination codon and the poly(A) addition site of the IAB^b gene, was treated with Klenow. This fragment was ligated into plasmid pTH-2 and used to transform MC1061 cells (7). After extensive restriction enzyme analysis, it was determined that plasmid pII-2SEC contained the predicted map, and this plasmid was used in subsequent experiments. Symbols: Regions encoding the H-2L^d gene; regions encoding IAB^b; 3' untranslated regions. Abbreviations and designations: Met, Metallothionein promoter; L, N, C₁, C₂, TM, I1, I2, and I3, regions of the protein encoded by exons of the H-2L^d gene; L, B₁, B₂, TM, I1, and I2, regions of the protein encoded by exons of the IAB^b gene.

isolated from A20 (Fig. 2, lane D). Furthermore, the protected fragment was about the size (85 nucleotides) predicted by comparison of the IAB^b cDNA sequence (28) to the genomic IAB^b sequence (5). These results demonstrate that splicing of the ligated IAB^b exon in the truncated H-2L^d mRNA occurred, similar to the splicing of the IAB^b mRNA normally expressed in a B-cell lymphoma, A20. The amino acid sequence of the COOH terminus of the truncated H-2L^d product is predicted in Fig. 3. The deleted gene product has removed 55 amino acids from the native H-2L^d gene product and replaced these with three new residues.

To demonstrate that the transfected gene directs the synthesis of a secreted antigen, the protein was isolated from

the cytoplasm and medium. Transformants TSEC, T111 (expressing the wild-type H-2L^d antigen), and DAP-3 cells (L cells) were metabolically labeled with [³⁵S]methionine. Immunoprecipitation of both cell lysates and culture medium was performed by using monoclonal antibody 30.5.7, which recognizes a determinant located on the C1 domain of the H-2L^d antigen (24). The immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both the wild-type and truncated H-2L^d antigens, expressed on transformants T111 and TSEC, respectively, appear to have been associated with β 2-microglobulin in the cytoplasm (Fig. 4B). When monoclonal antibody 30.5.7, which recognizes only H-2L^d antigens that have retained their native conformation, was used, no H-2L^d protein could

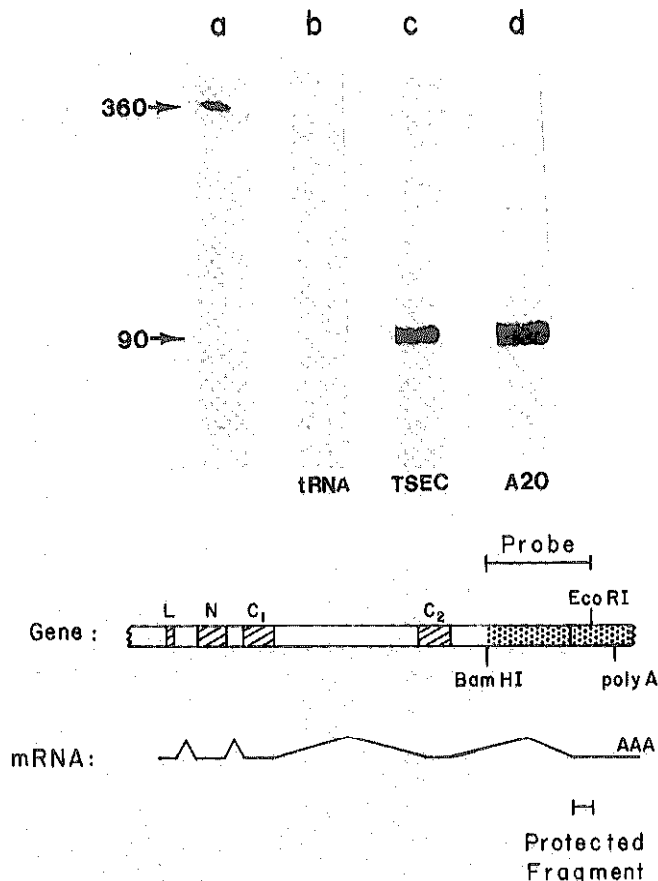


FIG. 2. Results of S1 nuclease mapping analysis of mRNA isolated from clone TSEC and B-cell lymphoma A20. A *Bam*HI-*Eco*RI fragment encoding the second cytoplasmic domain and 3' untranslated region of the IAB^b gene was cloned into M13mp9 and was used to isolate a radiolabeled probe, as described before (25). The single-stranded radiolabeled probe was hybridized with 3 μ g of total RNA from clone TSEC, 10 μ g of total mRNA of B-cell lymphoma A20, and 20 μ g of tRNA overnight at 55°C in a mixture containing 75% formamide. Each reaction mixture was then digested with 750 U of S1 nuclease at 42°C for 30 min, and the products were precipitated and analyzed by electrophoresis on denaturing polyacrylamide gels. The size of the protected fragment was measured at 90 bases by comigration with ³²P-labeled, *Hinf*-digested pBR322. (A) Probe fragment; (B) tRNA hybridized with the probe and digested with S1; (C) mRNA from clone TSEC hybridized with the probe and digested with S1; (D) mRNA from B-cell lymphoma A20 hybridized with the probe and digested with S1.

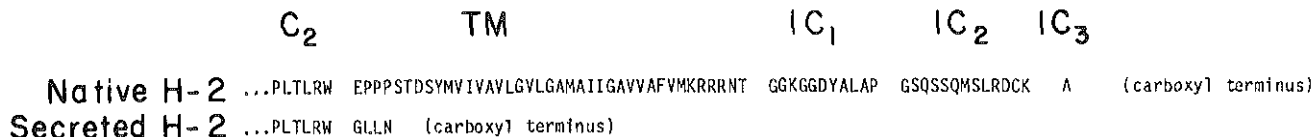


FIG. 3. Amino acid sequence of the C_2 , transmembrane, and cytoplasmic domains of the wild-type and secreted H-2L^d antigens.

be immunoprecipitated from the supernatant of cells expressing the wild-type H-2L^d antigen (Fig. 4A). However, culture medium from transformant TSEC, which expressed the truncated H-2L^d gene, contained a secreted H-2L^d antigen (Fig. 4A). As expected, the molecular size of the truncated H-2L^d antigen (39 kilodaltons) was significantly less than that of the wild-type protein (44 kilodaltons) (Fig. 4B). Secreted class I antigens have been detected before (8, 16, 19). The molecular size of naturally occurring secreted H-2 antigens (38,000 daltons) (8, 19) is similar to that of the secreted H-2 antigen described here.

No β_2 -microglobulin could be immunoprecipitated from the medium by using this monoclonal antibody. The absence of β_2 -microglobulin was probably caused by an exchange of radiolabeled active β_2 -microglobulin from fetal calf serum, as has been previously observed (3).

Membrane proteins were next labeled by lactoperoxidase-mediated iodination. No H-2L^d antigen could be immunoprecipitated from the cell membrane of TSEC cells, as is clearly shown in Fig. 4C. Thus, the truncated H-2L^d gene synthesized an H-2L^d antigen that was present in the cytoplasm and medium but was not detectable on the cell membrane.

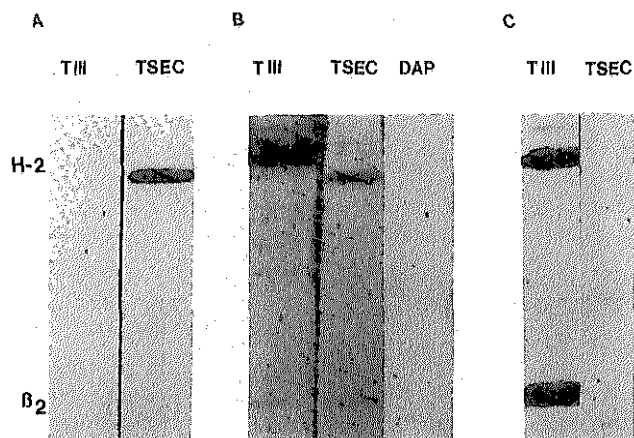


FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of radiolabeled H-2L^d antigens immunoprecipitated from the supernatant (A), cytoplasm (B), and cell surface (C) of transformants T111 and TSEC and DAP-3 cells. DAP-3 cells and transformants TSEC and T111 were labeled with 0.5 mCi of [³⁵S]methionine in 10 ml of methionine-free medium. After 5 h of incubation, the supernatant was removed, and the cells were trypsinized and lysed in 1 ml of 10 mM Tris hydrochloride (pH 8)-1% Nonidet P-40. Immunoprecipitation of 0.5 ml of supernatant was done essentially as described elsewhere, by using monoclonal antibody 30.5.7 (4). T111, TSEC, and DAP cells were also loosened from the flask with Versene (Whitaker) and iodinated with ¹²⁵I catalyzed by lactoperoxidase (13) and immunoprecipitated with monoclonal 30.5.7 (24). Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a vertical slab gel by the Laemmli procedure (17). Gels were soaked for 30 min in Enlightening (New England Nuclear Corp.), dried, and exposed overnight.

To study the kinetics of H-2L^d secretion, transformant TSEC was incubated in culture medium containing [³⁵S]methionine. Samples were taken at different time points and processed for immunoprecipitation. The antigen was first detectable in the medium after approximately 60 min of incubation (Fig. 5A), which was similar to the kinetics with which H-2 reaches the cell surface (18). The absolute amount of secreted, labeled H-2L^d antigen continued to increase to a maximum at about 8 h.

The stability of the secreted H-2L^d antigen was determined by adding supernatant containing [³⁵S]methionine-labeled H-2L^d back to the medium containing cultured cells. At different time points, samples were taken and processed for immunoprecipitation. No detectable degradation occurred after 9 h of incubation at 37°C in culture medium, although significant degradation was detectable after 27 h (Fig. 5B). It is possible, however, that the secreted [³⁵S]methionine-labeled H-2L^d antigen was protected from proteases by cold H-2L^d antigens secreted by transformant TSEC during the stability time course.

To determine whether a cell line secreting an H-2 antigen can serve as a target for CTL recognition, an *in vitro* allogeneic CTL assay was performed. CTLs generated by stimulating BALB/c (H-2L^d) spleen cells with C₃H (H-2^k) cells lysed all L cells (Table 1). C-H-2^{dm2} mice differ from BALB/c only in the expression of H-2L^d antigen. C-H-2^{dm2} mice express H-2K^d and H-2D^d and background antigens; therefore, CTLs from cultures of dm2 splenocytes stimulated with BALB/c cells are limited to recognizing the H-2L^d molecule. These effectors lysed all cells expressing the wild-type H-2L^d product, T111, and P815, a mastocytoma derived from the BALB/c mouse (Table 1). However, transformant TSEC, which secretes H-2L^d, was not recog-

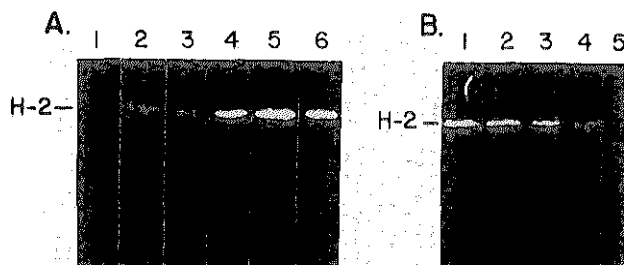


FIG. 5. Kinetics of secretion (A) and stability analysis (B) of H-2L^d secreted antigens. The rate of accumulation of secreted H-2L^d in the supernatants was measured by adding 1 mCi of [³⁵S]methionine to 10 ml of methionine-free medium. Samples (1 ml each) were removed after 30, 60, 120, 240, and 480 min and 27 h of incubation. The stability of the secreted antigen in the presence of cells in culture medium was determined as follows. Supernatant (5 ml) from cells radiolabeled with 1 mM [³⁵S]methionine was mixed with 5 ml of culture medium containing 1 mM methionine. Samples (1 ml each) were removed at 0, 2, 9, 27, and 69 h. All samples were immunoprecipitated with monoclonal 30.5.7, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and exposed overnight. (A) Lanes: 1, 30 min; 2, 60 min; 3, 120 min; 4, 4 h; 5, 8 h; 6, 27 h. (B) Lanes: 1, 0 min; 2, 2 h; 3, 9 h; 4, 27 h; 5, 69 h.

TABLE 1. T-cell-mediated lympholysis of normal and truncated H-2L^d genes expressed on L cells

Stimulated cells	% Specific release of ⁵¹ Cr ^d			
	P815 ^b (K ^d D ^d L ^d) ^c	T111 (L ^d K ^k D ^k)	DAP-3 (K ^k D ^k)	TSEC (secL ^d K ^k D ^k)
dm2 anti-BALB/c	99, 69, 68	50, 38, 24	18, 7, 12	17, 12, 11
BALB/c anti-C ₃ H	12, 5, 6	70, 57, 36	62, 45, 29	59, 55, 35

^a Effector-to-target-cell ratios 50:1, 25:1, and 10:1.

^b Target cells.

^c H-2 genes.

nized by H-2L^d-specific CTLs. Thus, the secreted H-2L^d molecule could not function as a target in H-2L^d allospecific killing. It is possible that secreted H-2L^d antigens block the T-cell receptor complex, thereby preventing recognition of the target cell. However, purified membranes or liposomes bearing the appropriate H-2 antigens do not have the ability to block lysis of targets by CTLs (22). Moreover, cells expressing the truncated H-2L^d gene do not have the capacity to block lysis of the appropriate target cells in cold target inhibition CTL assays (C. Reiss and S. Burakoff, unpublished results). Therefore, we believe that the H-2 antigen has to be a transmembrane protein to serve as a recognition element in CTL killing.

In summary, we have constructed a novel H-2L^d gene that synthesized a secreted H-2L^d molecule. The important conclusion from the experiments described here is that this truncated H-2L^d antigen was present in its native conformation, was associated with β 2-microglobulin, and was after secretion, relatively stable in culture medium. Further studies will determine whether large amounts of H-2L^d antigens are secreted by L cells or B-cell hybridomas transfected with this truncated H-2L^d gene. Eventually, secreted H-2 antigens might be a source of material that can be used in the elucidation of its tertiary conformation by X-ray crystallography (P. Bjorkman, J. L. Strominger, and D. C. Wiley, *J. Mol. Biol.*, in press).

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