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Secretion of a soluble class I molecule encoded by the Q10 gene of the C57BL/10 mouse

J.J.Devlin, A.M.Lew[1], R.A.Flavell and J.E.Coligan[1]

Biogen Research Corp., Fourteen Cambridge Center, Cambridge, MA 02142, and [1]Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 5, Room B1-04, Bethesda, MD 20205, USA

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The DNA sequence of the Q10 genes appears to be highly conserved amongst strains of mice and has only been found to be transcribed in the liver. An examination of the nucleotide sequence of the exon that normally encodes the transmembrane domain of class I molecules suggested that the Q10 gene encodes a secreted protein. We have established this by showing that L cells transformed with an expression vector containing the Q10 gene secrete a class I molecule which was identified with an antiserum raised against a peptide predicted by the Q10 transmembrane exon. Both the L cell-derived Q10 molecule and a class I protein immunoprecipitated from serum with this anti-peptide antiserum have mol. wts. of ~38 000; the Q10 molecule secreted by L cells is heterogeneous in mol. wt. This heterogeneity was drastically reduced after endoglycosidase F treatment, suggesting that Q10 molecules secreted into the serum by the liver may be glycosylated differently from those secreted by L cells. Endoglycosidase F treatment of both the L cell and serum forms of the soluble molecule yielded two products with mol. wts. of ~32 000 and 35 000; this is consistent with the observation that the predicted Q10 protein sequence has two potential glycosylation sites. In contrast to previous published results, the Q10 molecule reacted with rabbit anti-H-2 antisera which is consistent with its >80% homology to the classical transplantation antigens.

Key words: class I/Qa region/Q10 gene/secretion/histocompatibility

Introduction

Genes within the major histocompatibility complex (MHC) influence many biologically important functions, especially those associated with immune responses (Klein, 1979). A subset of these genes are the class I genes which in the mouse are located in the H-2 region and the Tla region on chromosome 17. The former encodes the classical transplantation antigens (H-2K, H-2D and H-2L); these function as recognition elements for cytotoxic T lymphocytes which play a pivotal role in allograft rejection and defence against virally infected cells. These antigens are found on most, if not all, cells. However, the vast majority of class I genes, 23 out of 26 in *b* haplotype mice (Weiss *et al.*, 1984) and 31 out of 36 in the *d* haplotype (Winoto *et al.*, 1983) belong to the Tla region which codes for the Qa and TL antigens. Although there is clearly significant structural homology between H-2 and Tla region-encoded class I molecules (Yokogama *et al.*, 1981; Soloski *et al.*, 1982), the functions of the latter have not been defined. It has been opined that they may be important in lymphocyte differentiation, as they have only been found on certain populations of lymphoid cells (Flaherty, 1981). The function of these molecules is one of the current mysteries of developmental biology and immunology.

DNA sequence analysis of class I genes and cDNA clones from this region have led to the discovery of several genes that have termination codons and codons for hydrophilic amino acids in the exon that normally encodes the transmembrane domain of class I molecules (Steinmetz *et al.*, 1981; Cosman *et al.*, 1982a; Mellor *et al.*, 1984); this leads to the suggestion that these genes might encode secreted class I proteins. In the SWR mouse, one of these genes has been found to be transcribed only in the liver and homologous genes (presumably the same gene or alleles) are transcribed in the liver of all other strains of mice that have been examined (Cosman *et al.*, 1982b). Additionally, an antiserum raised against a peptide predicted by the transmembrane exon of this SWR gene immunoprecipitated class I molecules from the serum and liver (Maloy *et al.*, 1984), thereby supporting the hypothesis that these unusual class I genes encoded secreted proteins.

To demonstrate that a class I gene can encode a secreted protein we have transformed murine L cells with an expression vector containing the Q10 gene from the C57BL/10 mouse (Weiss *et al.*, 1984). Mellor *et al.* (1984) have shown that this gene is 99.4% homologous on the nucleotide level with the SWR gene that putatively encodes a secreted class I molecule; in particular, these two genes are identical in the exon that normally encodes the transmembrane domain of class I proteins. To overcome the reported tissue-specific transcription of the Q10 gene we have placed it under the control of the mouse methallothionein I promoter. Here we show that the protein encoded by the Q10 gene is secreted by L cells transformed with this construct.

Results

In an initial attempt to obtain expression of the Q10 gene in L cells, we co-transformed a plasmid containing the entire Q10 gene and ~7 kb of 5′-flanking DNA into TK^- L cells along with pOPF (TK^+). However, we were unable to detect Q10-specific RNA in any of the 12 HAT-resistant colonies examined. Since it had been previously reported that a SWR gene highly homologous to the C57BL/10 Q10 gene was transcribed in the liver but not in the thymus, spleen, kidney or testis (Cosman *et al.*, 1982b), we felt that the Q10 gene might be controlled in a tissue-specific manner that prevented expression in L cells. This would be consistent with the report that the transfer of fibroblast chromosomes into hepatoma cells can repress the synthesis of liver-specific products (Killary and Fournier, 1984). To circumvent this possible problem, we constructed a plasmid with the promoter and 5′-flanking region of the mouse metallothionein-I gene immediately upstream of the Q10 coding region (Figure 1). The metallothionein gene has a strong promoter that has been used successfully in the expression of human growth hormone in mice (Palmiter

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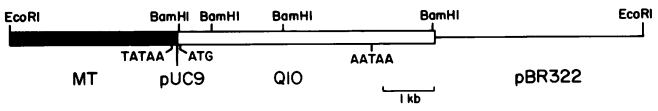

Fig. 1. Construction of pM10. The black box represents the promoter region of the mouse metallothionein-1 gene. The 26 bp from pUC9 are indicated by the long vertical line. The open box indicates DNA containing the Q10 gene, and the thin line indicates pBR322 DNA. The locations of the TATAA box of the metallothionein promoter, the ATG corresponding to the initiation codon of the Q10 gene and the poly(A) addition signal (AATAAA) are shown.

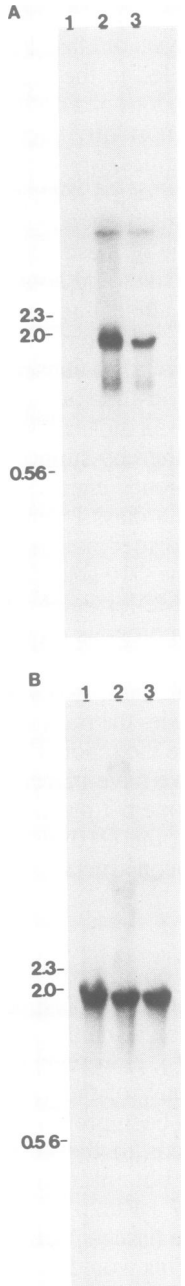

Fig. 2. Detection of the Q10 message in transformed cells. Blots with RNA from colonies TK, 3 and 13 in **lanes 1, 2** and **3**, respectively, were hybridized with the following probes. **(A)** The probe from pPst2D (specific for transcripts of the Q10 gene and its alleles). **(B)** The probe from the 3′ half of the K^b gene (cross-reactive with all class I transcripts). The positions and sizes in kb of mol. wt. markers derived from a *Hind*III digestion of the phage λ are indicated on the left of each blot.

et al., 1983). The *Bgl*II site in the metallothionein gene used in the construction of pM10 is 64 bp downstream from the mRNA cap site; assuming transcription starts in the same place, the ATG corresponding to the initiation codon in pM10 would be 100 bp downstream from the cap site.

After transformation of L cells with this construct and the establishment of cell lines from HAT-resistant colonies, we examined transcription of the Q10 gene by RNA blot analysis. Cosman *et al.* (1982b) have reported that a *Pst*I fragment from the 3′-untranslated region of a cDNA clone (pPst2D), which was derived from the apparent allele of the Q10 gene in the q haplotype, could be used to detect Q10-like transcripts from a number of different strains of mice. As can be seen in Figure 2A, this probe detects a mRNA of appropriate size from the cell lines 3 and 13 but not from the L cell line that had been transformed with the TK gene alone. On an identical blot, a probe consisting of the 3′ *Bam*HI fragment of the H-$2K^b$ gene, which includes the exon encoding the highly conserved third external protein domain (Weiss *et al.*, 1983), hybridizes to bands of roughly equal intensity in the RNA from all three cell lines (Figure 2B). Since this probe should hybridize to all class I messages, this result shows that the

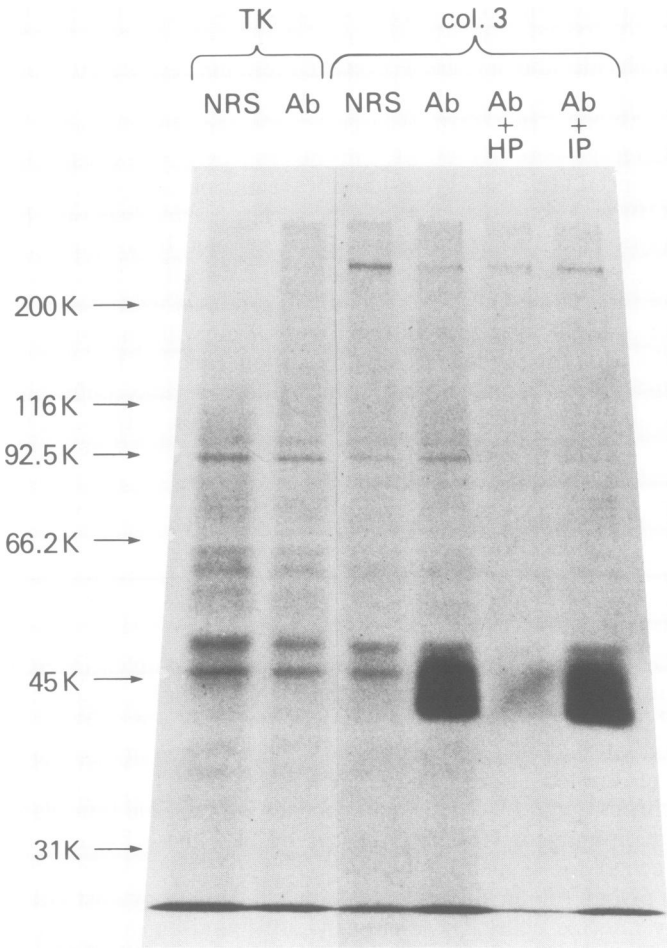

Fig. 3. Immunoprecipitation of culture supernatants with anti-peptide serum. Culture supernatants from ^{35}S-labeled transformed cells (colony 3) and control cells (TK) were reacted with normal rabbit serum (NRS), anti-peptide serum (Ab), anti-peptide serum plus excess homologous peptide (Ab + HP), anti-peptide serum plus irrelevant peptide (Ab + IP). The immune complexes were precipitated with protein A agarose beads and analyzed by SDS-PAGE, followed by fluorography.

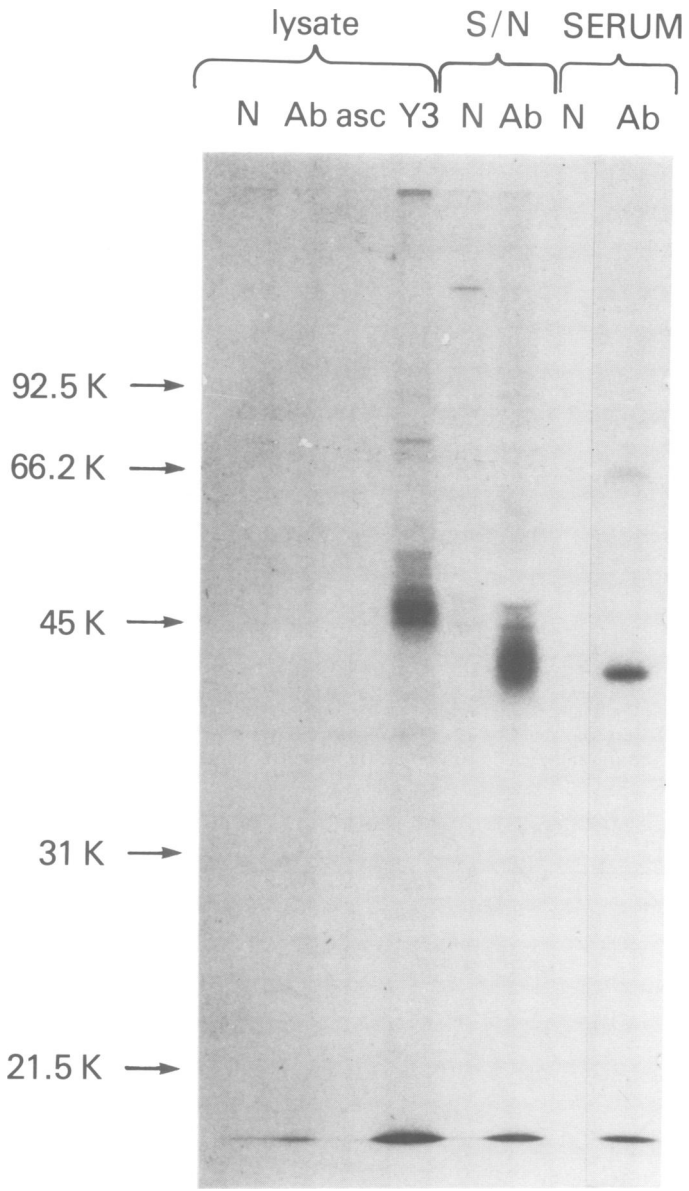

Fig. 4. Comparison of the soluble class I molecule secreted by transformed cells with that found in mouse serum. Culture supernatant (S/N) and cell lysates (lysate) of ^{35}S-labeled transformed cells and serum from mice injected with [^{35}S]methionine (serum) were immunoprecipitated with normal rabbit serum (N), anti-peptide serum (Ab), control mouse ascites (asc) or a monoclonal antibody against H-2K^b (Y3). Y3 also reacts with H-2K^k.

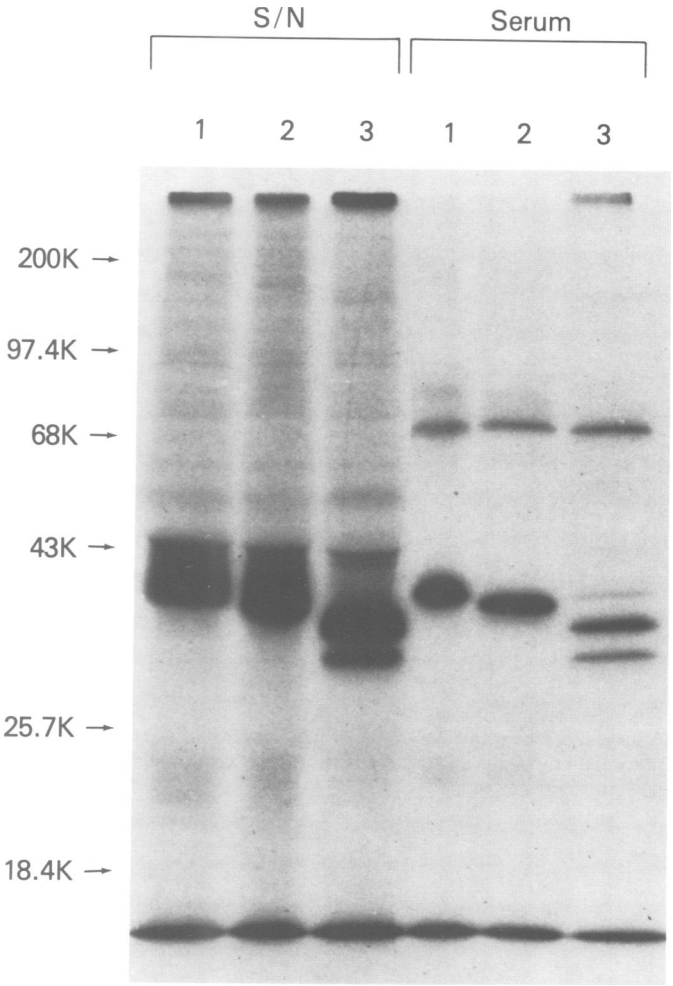

Fig. 5. The effect of glycosidase treatment on the soluble class I molecule. SDS-PAGE analysis of ^{35}S-labeled culture supernatant (S/N) and mouse serum (serum) immunoprecipitated with anti-peptide serum without enzyme treatment **(lane 1)** after treatment with neuraminidase **(lane 2)** and after treatment with endo F **(lane 3)**.

failure of the Q10-specific probe to hybridize to RNA from colony TK is not due to trivial factors such as degradation of RNA.

Immunoprecipitation of the soluble class I molecule from the culture supernatant of transfected cells

Rabbit antiserum was prepared against a synthetic dodecapeptide which corresponds to a segment of the transmembrane domain peculiar to the soluble molecule (Maloy *et al.*, 1984). Several bands with a mol. wt. of ~38 000 and a 12 000 mol. wt. band [presumably β_2-microglobulin (β_2m)] were precipitated from supernatant of Q10-transformed cells with this antiserum (Figure 3). Specificity was confirmed by lack of precipitation by control rabbit serum and by inhibition by excess homologous peptide. The presence of an irrelevant peptide did not produce inhibition. The above bands were not immunoprecipitated from the supernatant of the control cells (TK). Figure 4 shows that the major band immunoprecipitated from the supernatant corresponds in mol. wt. to the soluble class I molecule immunoprecipitated from serum. The molecule was not detected in the lysates of transformed cells, although clearly these cells contained the classical H-2 molecule (Figure 4). The heterogeneity in the immunoprecipitates of the secreted products of the transformed cells is partly reduced by neuraminidase and further reduced by endoglycosidase F (endo F) to essentially three bands, thus indicating that the heterogeneity is largely due to differential glycosylation (Figure 5).

Detection of the soluble class I molecule by Western blotting

The results of Western blotting (Figure 6) confirm those found by immunoprecipitation. Again, only the supernatant of transformed cells contained the soluble molecule. A broader band of staining was found in the supernatant than that found in serum.

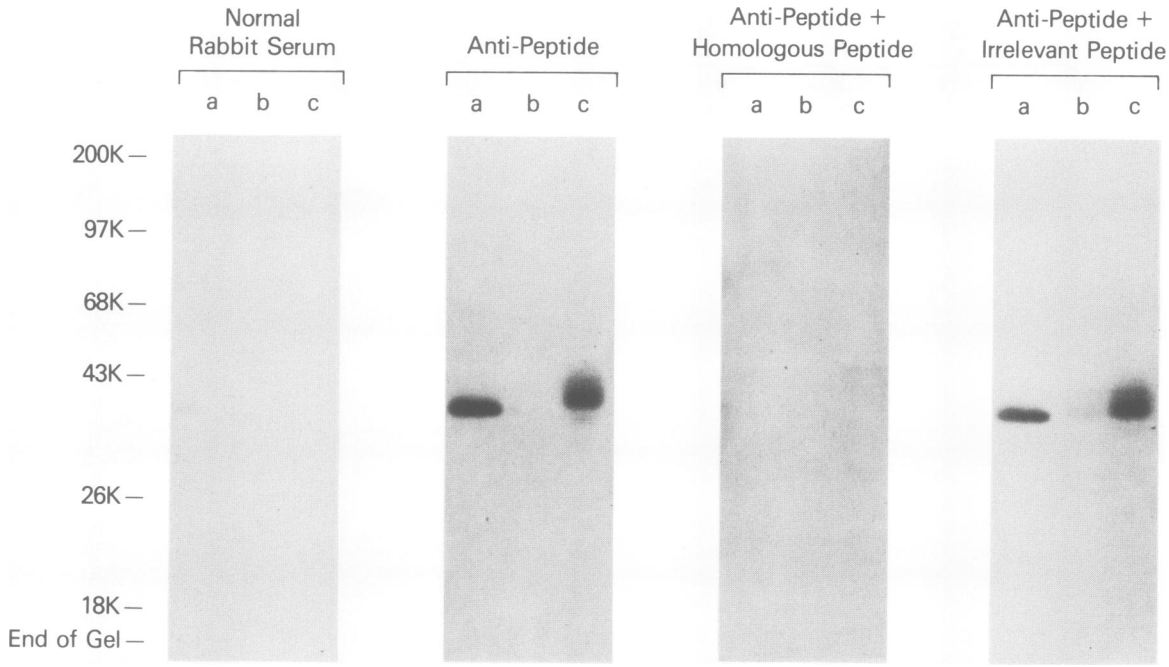

Fig. 6. Detection of the soluble class I molecule on a Western blot. Ammonium sulphate precipitated proteins from mouse serum **(lane a)**, from culture supernatants of control cells **(lane b)** and of transformed cells **(lane c)** were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose filter which was reacted with normal rabbit serum, anti-peptide serum, anti-peptide serum plus homologous peptide or anti-peptide plus an irrelevant peptide.

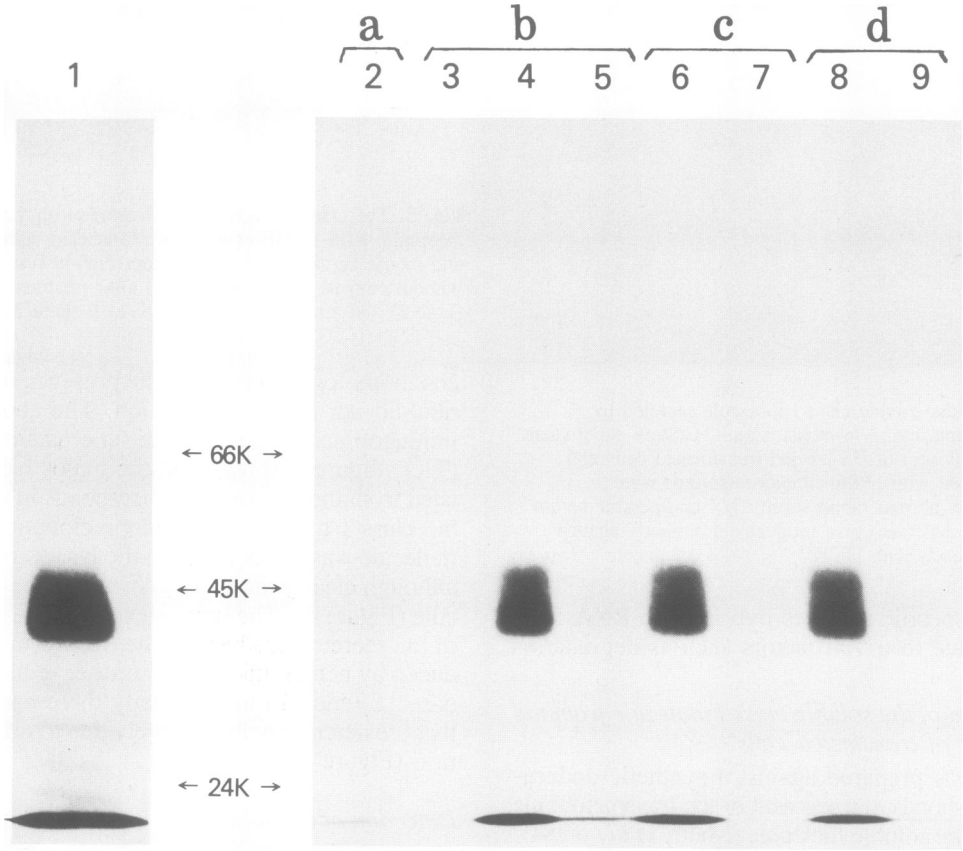

Fig. 7. Reactivity of secreted molecule with rabbit anti-H-2 and anti-mouse β_2m sera. The soluble molecule was purified from ^{35}S-labeled culture supernatant by peptide elution from an anti-peptide immunoadsorbent column. **Lane 1** shows the fluorograph of the purified material after SDS-PAGE. Immunoprecipitation of this material was performed with the following sera **(a)** normal rabbit serum **(lane 2)**, **(b)** anti-peptide serum after pre-clearance by rabbit anti-β_2m **(lane 3)**, after pre-clearance by normal rabbit serum **(lane 4)**, or after pre-clearance by rabbit anti-H-2a **(lane 5)**, **(c)** anti-H-2a serum after pre-clearance by normal rabbit serum **(lane 6)**, or after pre-clearance by anti-peptide **(lane 7)**, **(d)** anti-β_2m serum after pre-clearance by normal rabbit serum **(lane 8)**, or after pre-clearance by anti-peptide **(lane 9)**.

Association with β_2m and cross-reaction with classical H-2 antigens

The soluble molecule, which was purified by affinity chromatography on an anti-peptide column, could be immunoprecipitated with a broad specificity rabbit anti-H-2 and rabbit anti-β_2m serum (Figure 7, lanes 6 and 8). Pre-clearance with anti-peptide serum negates this (lanes 7 and 9). Conversely, pre-clearance with either rabbit anti-H-2 or anti-β_2m removes the molecule that is precipitable with anti-peptide serum. This indicates that the soluble molecule is non-covalently bound to β_2m and that the heavy chain of ~38 000 daltons has some epitopes in common with classical H-2 molecules.

Discussion

Previous studies have shown that mouse serum and liver contain a class I-like protein which can be immunoprecipitated by an antiserum raised against a peptide predicted by the DNA sequence of a cDNA clone from the SWR/J mouse (H-2^q) (Maloy *et al.*, 1984). Here we have shown that mouse L cells (H-2^k) transformed with an expression vector containing the Q10 gene from the C57BL/10 mouse (H-2^b) secrete a similar soluble class I protein.

After immunoprecipitation of the secreted proteins with the anti-peptide antiserum, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicates that the mol. wt. of the Q10 gene product is ~38 000, which is similar to the serum form of the molecule (Figure 4). However, compared with the serum form of the Q10 gene product, the molecule expressed by L cells is considerably more heterogeneous in size; a substantial amount of the L cell products being larger. Treatment of the L cell secreted Q10 gene products with glycosidases significantly reduces the heterogeneity. The serum form and the L cell secreted Q10 gene product are identical in size after endo F treatment. This is strong evidence that the class I glycoprotein immunoprecipitated from the serum was encoded by the Q10 gene. The different glycosylation of the Q10 gene product by L cells and liver is not surprising as other proteins are known to be glycosylated differently in different cell types, e.g., class II molecules (Cullen *et al.*, 1981). Alternatively, it is possible that the glycosyl residues of the Q10 gene product are further processed during circulation in the blood; such processing might not occur in the tissue culture media used to support growth of the L cells.

Prior to endo F treatment, the serum form of the Q10 gene product, as well as the bulk of the L cell secreted form, has a mol. wt. of 38 000 (Figure 3). After treatment with the enzyme, most of the material is located at a mol. wt. position of ~35 000 indicative of the loss of a single N-linked glycosyl unit. A significant portion of the material occurs in a second band at ~32 000 which is consistent with the loss of two N-linked glycosyl units. These data are consistent with the fact that the Q10 gene product has two N-linked glycosylation sites, positions 86 and 256 (Kress *et al.*, 1983). A deglycosylated mol. wt. of 32 000 is also consistent with the anticipated mol. wt. of the 311 residue Q10 gene product.

Previously it had been reported that, in addition to lacking reactivity with broad specificity anti-H-2 alloantisera (Kress *et al.*, 1983), the Q10 gene product lacked reactivity with rabbit xenoantisera against purified H-2^a molecules (Maloy *et al.*, 1984). Since the Q10 gene appears to be non-polymorphic (Mellor *et al.*, 1984), the lack of alloreactivity is not surprising. However, since the soluble molecule is >80% homologous with membrane-bound H-2 molecules (Kress *et al.*, 1983) the reported lack of reactivity with xenoantisera was surprising. In the present study, we were clearly able to demonstrate that the Q10 gene product does react with rabbit xenoantiserum. Since the source of the rabbit xenoantiserum was the same, the reason for the previous failure to detect this reactivity is unknown. As expected, the Q10 gene product was found to be non-covalently associated with β_2m antiserum.

The function of the Q10-derived secreted class I molecule remains to be elucidated. Because of its liver-specific transcription and reports that liver transplants induce donor-specific unresponsiveness to further tissue transplants, it has been suggested (Kress *et al.*, 1983) that the Q10 antigen is the serum factor that mediates this unresponsiveness. However, it is not clear how an apparently non-polymorphic molecule, as judged by the 99.4% sequence homology of the Q10 gene from C57BL/10 and SWR strains of mice (Mellor *et al.*, 1984), could mediate this unresponsiveness unless a donor-specific molecule was also involved. Nevertheless, to provide clues to the function of this soluble class I molecule, we are utilizing the material secreted by L cells to develop a reliable, quantitative assay to ascertain the normal serum levels and turnover rate of this molecule in adult mice. Once normal adult levels are known, we will investigate the level at various states of development and during various perturbations of body function such as pregnancy and graft recipiency.

A major obstacle in functional and structural (X-ray crystallography) studies on class I molecules has been the availability of material. In the case of the soluble class I molecule, mouse serum is expensive, may contain more than one type of soluble molecule and has a formidable amount of irrelevant protein which would hinder purification. Purified Q10 antigen from the L cell lines would also allow us to investigate its effect on *in vivo* and *in vitro* assays of immunological functions, e.g., T cell cytotoxicity.

Materials and methods

Plasmid construction

All enzymes were used in accordance with the manufacturers instructions (New England Biolabs, Beverly, MA). In order to construct pM10 we started with a plasmid containing the Q10 gene on a 12-kb *Eco*RI fragment. This gene has been isolated from the C57BL/10 mouse (Weiss *et al.*, 1984). After partial digestion with *Bam*HI and complete digestion with *Eco*RI, we isolated the fragment beginning at the *Bam*HI site located between the Q10 promoter and the ATG corresponding to the initiation codon and ending at the *Eco*RI site following the Q10 poly(A) addition site. This fragment was ligated to pUC9, which had been digested with *Bam*HI and *Eco*RI. *Escherichia coli* were transformed and a colony containing a plasmid consisting of the *Bam*HI-*Eco*RI fragment inserted into pUC9 was isolated. The Q10 gene fragment was recovered from this plasmid by digestion with *Hind*III and *Eco*RI (the *Hind*III site in pUC9 is close to the *Bam*HI site; therefore, the fragment now had 26 bp from the pUC9 polylinker added to the 5′ end). We then used the large fragment of DNA *Pol*I (Klenow) to fill in the protruding 5′ ends, making this fragment blunt ended.

The plasmid pMK (gift of Richard Palmiter) was digested with *Bgl*II and *Bam*HI. We then isolated a fragment comprising the large *Eco*RI-*Bam*HI fragment of pBR322 and a 3.2-kb fragment derived from the region just upstream of the ATG corresponding to the initiation codon of the mouse metallothionein-I gene. This fragment was also made blunt ended with Klenow, ligated to the blunt ended fragment containing the promoterless Q10 gene and the mixture used to transform *E. coli*. A plasmid containing the metallothionein promoter and Q10 gene in the correct orientations was isolated and termed pM10 (Figure 1).

L cell transformation

L cells lacking the enzyme thymidine kinase were co-transformed with a ten-to-one molar ratio of either pM10 or a subclone of the Q10 gene and

pOPF, which contains the thymidine kinase gene and HAT-resistant colonies were selected as described by Wigler *et al.* (1979). Colonies 3 and 13 are described in this paper. Colony TK was isolated after transformation with pOPF only.

RNA analysis

Total RNA was prepared by guanidine isothiocyanate extraction and transferred to nitrocellulose as described by Maniatis *et al.* (1982). The integrity of the RNA was checked by examining the rRNA bands in an ethidium bromide stained agarose urea gel. 10 μg of total RNA from each cell line was electrophoresed in 1% agarose containing formaldehyde and then transferred to nitrocellulose.

The filters were pre-hybridized for 1 h at the appropriate temperature. The solution used for both pre-hybridization and hybridization was: 5 x SSC, 50 mM Na_3PO_4 (pH 7.0), 10 x Denhardts, 0.1% SDS, 10% dextran sulfate and 100 μg/ml tRNA. The filters were hybridized at 65°C for 18 h and then washed at 65°C with 0.1 x SSC in 0.1% SDS. The restriction fragments used as probes were isolated from gels and labeled by nick translation to a specific activity of $>10^8$ c.p.m./μg of DNA.

Metabolic labeling and immunoprecipitation

For *in vitro* labeling, confluent L cells in roller bottles were incubated for 3 days in minimum essential medium in Hanks salts containing 10% fetal calf serum and 10 μCi [^{35}S]methionine (Amersham, sp. act. 1000 Ci/mmol) per ml. Cells were lysed by 1% NP-40 detergent (Schwartz and Nathenson, 1971). Culture supernatant or the soluble fraction of the cell lysate (200 μl) was pre-cleared with 50 μl v/v formalin treated *Staphylococcus aureus* Cowan 1 (prepared by the method of Kessler, 1975) in NETT buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris, 1% Triton X-100 and 0.02% NaN_3, pH 8.0). The pre-cleared material was reacted for 1 h at room temperature with 20 μl of antiserum. Samples were then incubated with 40 μl of 50% v/v protein-A agarose (Bethesda Research Laboratories, Gaithersburg, MD) in NETT for 1 h at room temperature with mixing. The agarose beads were washed four times with NETT. The bound proteins were analyzed by SDS-PAGE under reducing conditions followed by fluorography (Aley *et al.*, 1984). Rabbit anti-peptide sera were raised according to Maloy *et al.* (1984). A broad specificity xenoantiserum against H-2 heavy chain and a rabbit anti-mouse β_2m antiserum were generous gifts of Dr M.J.Rogers, NIH.

For *in vivo* labeling, female C57BL/10J mice were injected i.v. with 1 μCi [^{35}S]methionine each and serum pooled from 6 and 24 h bleeds was immunoprecipitated as above except that 400 μl of 50% v/v staphylococci were used for pre-clearance.

Western blotting

Serum and culture medium were fractionally precipitated by half saturated ammonium sulphate. The precipitates were dialyzed in 0.01 M phosphate buffered saline (PBS) pH 7.4, run on SDS-PAGE under reducing conditions and electrophoretically transferred to nitrocellulose filters. The filters were reacted with rabbit antiserum diluted 1:100 in PBS containing 0.1% Tween 20 for 2 h, washed three times for 10 min in PBS, followed by 2 μg/ml protein A peroxidase (Sigma Chemical Co., St. Louis, MO) in PBS containing 0.1% Tween 20 for 2 h. After another three washings in PBS, diaminobenzidine tetrahydrochloride at 0.05% in 0.05 M citrate/phosphate buffer pH 5.0 containing 0.01% H_2O_2 was used as chromogen.

Glycosidase digestions

Endo F digestion was performed according to Elder and Alexander (1982). Samples were heated for 15 min in 0.1 M sodium phosphate buffer pH 6.1 containing 70 mM EDTA, 0.1% SDS and 1% 2-mercaptoethanol. NP-40 detergent was added to a final concentration of 1% and 10 μl of Endo F (New England Nuclear, Boston, MA) was added. The reaction was allowed to proceed at 37°C for 2 h.

Neuraminidase (Sigma) digestion was performed according to Cowan *et al.* (1982).

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