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Immunology

Identification of a second class I antigen controlled by the *K* end of the *H-2* complex and its selective cellular expression

(tunicamycin)

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ABSTRACT **Immunoprecipitates obtained from [^{35}S]methionine-labeled spleen cells by using monoclonal antibodies specific for H-2K^d and H-2D^d have been separated by two-dimensional polyacrylamide gel electrophoresis. Analysis of these gel patterns revealed the presence of an additional product of the *K* end of the *H-2*^{d} complex, designated here as H-2K′. To determine whether H-2K′ is a unique protein or a differentially glycosylated form of the previously characterized H-2K^d histocompatibility antigen, nonglycosylated molecules labeled in the presence of tunicamycin were examined. The results showed that both H-2K and H-2K′ have distinct nonglycosylated polypeptide precursor forms. The approximate molecular weight differences between the fully glycosylated and nonglycosylated molecules also indicated the presence of three oligosaccharide side chains on H-2K′, as is the case with H-2K^d, whereas H-2D^d has only two oligosaccharide units. The cellular expression of H-2K′ was also investigated. Comparison of H-2 antigens immunoprecipitated from normal spleen cells and from thioglycollate-induced adherent peritoneal exudate cells cultured in the presence or absence of supernatant fluids from concanavalin A-stimulated spleen cells revealed that H-2K′ was not expressed on the adherent peritoneal cells. This indicates that H-2K′ is expressed in a tissue-specific manner, unlike the classical histocompatibility antigens H-2K and H-2D.**

The *K* and *D* regions of the murine *H-2* major histocompatibility complex encode the classical serologically defined histocompatibility antigens that are present on all nucleated somatic cells. The gene products of the *H-2K* and *H-2D* loci have the common structure of class I major histocompatibility complex antigens, existing as 45,000-dalton integral membrane glycoproteins noncovalently associated with a 12,000-dalton polypeptide, β_2-microglobulin. These antigens evoke the strongest immune response during allograft rejection and are responsible for the restriction of cytotoxic effector T cells involved in the lysis of target cells bearing foreign antigens, such as viral determinants (1).

Although it was originally thought that the *K* and *D* regions each controlled only a single gene product (2), recent serological studies have suggested that the *K* and *D* regions of several haplotypes encode more than one class I antigen (3–6). In addition, analysis of a library of cosmid clones places the number of unique class I genes between 30 and 40 (7), of which only a few have been identified biochemically. These include the classical histocompatibility antigens, which include products of the *H-2K* and *H-2D* loci and have been identified in all *H-2* haplotypes, as well as products of the *H-2L* and *H-2R* genes which are controlled by the *D* region of *d* and *q* haplotypes (4, 8, 9). Other *H-2*-linked class I antigens included in the estimated number of class I genes are the selectively expressed differentiation antigens Qa-1, Qa-2, and TL (10) which map to the right of the *D* region. Thus far, only one product of the *K* region has been defined biochemically; however, Démant and his colleagues have reported serological evidence from cocapping experiments for the existence of a second K^d region antigen (11, 12).

In this investigation, analysis of H-2 immunoprecipitates from various mouse strains by two-dimensional gel electrophoresis has led to the identification of a second protein controlled by the *K* end of the *H-2*^{d} complex, H-2K′. Comparison of the tissue distribution of H-2K and H-2K′ reveals that H-2K′ is not expressed on adherent peritoneal exudate cells (PEC), although both antigens are present on splenic lymphocytes.

MATERIALS AND METHODS

Mice. C57BL/10 (B10), B10.D2, B10.A, and B10.A(3R) mice were obtained from H. O. McDevitt (Stanford University School of Medicine, Stanford, CA). C3H, BALB/c, and B10.GD mice were bred in our animal facility.

Antisera. The MK-S4 (anti-I-A^s) hybridoma cell line was a gift from P. Marrack and J. Kappler (University of Colorado Medical School, Denver, CO). The 10-3.6 and 10-2.16 (anti-I-A^k) hybridoma cell lines were developed in the laboratory of L. A. Herzenberg (Department of Genetics, Stanford University Medical School) and have been described (13). The 34-1-2 (anti-H-2K^d,D^d,K^b,r,s,p,q) (14) and 20-8-4 (anti-H-2K^b,D^b,K^d,r,s) (15) hybridoma cell lines were generously provided by D. Sachs and K. Ozato (National Cancer Institute). Monoclonal antibodies were used as hybridoma culture supernates.

Preparation and Radiolabeling of Cells. *Splenic lymphocytes.* The preparation and radiolabeling of splenic lymphocytes were performed as described (16, 17). Briefly, spleen cells were incubated in 60-mm plastic tissue culture dishes (Falcon) at 2.5 × 10^7 cells per ml in methionine-free RPMI-1640 medium (GIBCO) containing 5% newborn calf serum (NCS) (Irvine Scientific) and [^{35}S]methionine (250 μCi/ml; 600–1,000 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq; Amersham). After 5 hr in culture the nonadherent cells were harvested.

Adherent peritoneal cells. Unimmunized BALB/c mice were injected intraperitoneally with 1.5 ml of 3% thioglycollate medium (Difco). Four days later, the peritoneal cells were harvested by flushing the peritoneal cavity with serum-free RPMI-1640 medium containing 10 units of heparin per ml. The resulting PEC were recovered by centrifugation and washed once in RPMI-1640 medium without heparin. The erythrocytes were then lysed with hemolytic Gey solution containing NH_4Cl. The remaining cells were washed, resuspended in RPMI-1640 containing 15% NCS (complete medium), and plated on 100-mm plastic tissue culture dishes (Corning) at a concentration of 10

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Abbreviations: B10, C57BL/10; PEC, peritoneal exudate cells; NCS, newborn calf serum; Con A, concanavalin A.

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$\times 10^6$/ml of medium. After incubation at 37°C in an atmosphere of 7% CO_2/93% air for 6 hr, nonadherent cells were removed by washing the monolayers twice with complete medium. The adherent cells were then returned to culture for an additional 18 hr. At the end of the 24-hr period, any residual nonadherent cells were removed by two additional washes of the cell monolayer. The cells were then returned to culture at 37°C for an additional 48 hr in RPMI-1640 medium containing 10% NCS. To increase H-2 antigen expression on cultured PEC, some cells were incubated in medium containing 20% (vol/vol) supernatant fluids from concanavalin A (Con A)-stimulated mouse cells, as described (18). An equivalent amount of Con A added to normal culture medium served as the control supernatant.

After 48-hr incubation, adherent PEC were removed from the tissue culture dishes by incubating the monolayers with phosphate-buffered saline containing EDTA according to the procedure described by Stern *et al.* (19). Cells were recovered by centrifugation, and 2–5 $\times 10^7$ cells were returned to culture for an additional 5 hr in methionine-free medium containing 5% NCS and 250 μCi of [^{35}S]methionine per ml as described for mouse spleen cells. At the end of the labeling period, the cells were recovered with a rubber policeman.

Tunicamycin Treatment. The tunicamycin, obtained from Carol Sibley (University of Washington, Seattle), was originally a gift to her from Robert Hamill (Eli Lilly). From a 1 mg/ml stock solution, tunicamycin was added to splenic lymphocytes in methionine-free RPMI-1640 labeling medium (2.5 $\times 10^7$ cells per ml) to a final concentration of 15 μg/ml. The cells were incubated in an atmosphere of 7% CO_2/93% air at 37°C for 1 hr prior to the addition of [^{35}S]methionine at 250 μCi/ml. Radiolabeling was continued in the presence of tunicamycin for an additional 5 hr.

Detergent Extraction and Immunoprecipitation of Antigens. These steps were performed as described (17). Briefly, washed, radiolabeled cells were extracted at 10^8 cells per ml for 20 min at 4°C in extraction buffer containing 0.5% Nonidet P-40 detergent (Particle Data, Elmhurst, IL). Cell extracts were cleared for 20 min at 4°C with heat-killed, formalin-fixed *Staphylococcus aureus*, Cowan strain I (IgSorb, The Enzyme Center, Boston) and then centrifuged to remove the bacteria. The H-2 antigens were immunoprecipitated by addition of 300 μl of hybridoma culture supernate to 100 μl of extract. After a 30-min incubation at 4°C, *S. aureus* were added. The bound antigens were eluted from the bacteria by addition of 50 μl of isoelectric focusing sample buffer per immunoprecipitate.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Two-dimensional polyacrylamide gel electrophoresis was done as described (17). Immunoprecipitated proteins were separated according to charge in the first dimension by using non-equilibrium pH gradient electrophoresis (NEPHGE) in tube gels. The second dimension was size separation on 10% NaDodSO$_4$/polyacrylamide slab gels. For fluorography, fixed and stained slab gels were rinsed in water for 30 min, treated with 1 M sodium salicylate for 30 min, dried, and then exposed to preflashed Kodak XAR-5 X-Omat film.

RESULTS

Identification of a Second H-2K^d Protein. Analysis of H-2 antigens by two-dimensional polyacrylamide gel electrophoresis has demonstrated that the *K* and *D* regions control a heterogeneous population of molecules with respect to molecular weight and pI (9, 16). Part of this heterogeneity is the result of post-translational modifications, such as glycosylation of cytoplasmic precursor molecules (9, 20). However, the existence of multiple gene products can also contribute to the observed molecular heterogeneity, as has been observed in the case of *D* region-controlled products H-2L^d, H-2R^d, and H-2M^d (4, 8, 21).

Our analyses of the two-dimensional gel patterns generated by monoclonal antibodies specific for class I molecules of the *d* haplotype have led to the identification of a second protein controlled by the *K* end of the *H-2^d* complex, which we have given the provisional designation H-2K′. Fig. 1 shows the gel patterns of immunoprecipitated proteins from [^{35}S]methionine-labeled spleen extracts from several mouse strains. The gel of antigens immunoprecipitated from B10.D2 (*K^{d}D^d*) spleen cells by monoclonal antibody 34-1-2 (anti-H-2K^d, D^d) shows the spots corresponding to the previously recognized H-2K^d and H-2D^d products (Fig. 1g) (16). The cytoplasmic precursor (cK, cD) and cell surface (sK, sD) forms have been identified in experiments utilizing selective cell surface radiolabeling with ^{125}I and pulse–chase experiments (16).

Of interest in the current investigation was the presence of the additional group of spots labeled K′. The cytoplasmic spot, cK′, was slightly more acidic and of lower molecular weight than the spots corresponding to cK, the cytoplasmic forms of the H-2K protein. The corresponding cell surface forms of H-2K′, labeled sK′, appeared directly below the more acidic forms of the

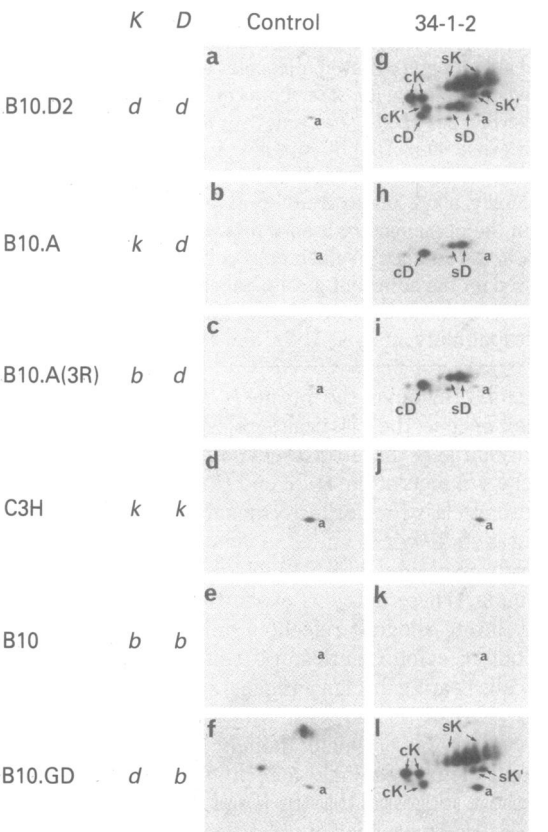

FIG. 1. Identification of a second *K* end-controlled protein, H-2K′, by two-dimensional polyacrylamide gel electrophoresis. Proteins extracted from [^{35}S]methionine-labeled splenic lymphocytes of various genetically defined mouse strains were immunoprecipitated with 300 μl of hybridoma culture supernate containing the following monoclonal antibodies: MK-S4, anti-I-A^s (*a–e*); 10-3.6, anti-I-A^k (*f*); and 34-1-2, anti-H-2K^d,D^d (*g–l*). After the separation, the fluorograms shown here were prepared. The cytoplasmic precursor (cK, cK′, cD) and mature cell surface (sK, sK′, sD) forms of the H-2 antigens are labeled. The basic end of the gel is at the left and the acidic end is at the right. a, Actin. The *H-2* haplotypes of the strains tested were as follows: B10.D2 (*H-2^d*), B10.A (*H-2^a, K^k A^k B^k J^k E^k C^d S^d D^d*), B10.A(3R) (*H-2^{i3}, K^b A^b B^b J^b E^k C^d S^d D^d*), C3H (*H-2^k*), B10 (*H-2^b*), B10.GD (*H-2^{g2}, K^d A^d B^b J^b E^b C^b S^b D^b*).

sK product. Our assignment of cytoplasmic and surface forms of H-2K′ is consistent with previous results from our laboratory obtained with samples extracted with the detergent Lubrol WX, which solubilizes the integral membrane glycoproteins of the plasma membrane but allows the glycoproteins associated with the endoplasmic reticulum to remain primarily in the Lubrol WX-insoluble fraction (22). In those studies, the sK′ spots were observed in the two-dimensional gel pattern of Lubrol-solubilized fractions and the cK′ spots were seen in conjunction with the Lubrol WX-insoluble fraction.

To determine whether the H-2K′ protein maps to the *K* end or *D* end of the *H-2* complex, two-dimensional polyacrylamide gel electrophoresis was performed on B10.A (K^kD^d) and B10.A(3R) (K^bD^d) immunoprecipitates obtained by using monoclonal antibody 34-1-2. Immunoprecipitates from C3H (K^kD^k) and B10 (K^bD^b) served as additional controls. Monoclonal antibody 34-1-2 was reported to react weakly by cytotoxicity with H-2K^b (14); its reactivity apparently is not strong enough to allow it to precipitate H-2K^b from B10 and B10.A(3R) extracts. From the gels shown in Fig. 1, it is clear that H-2K′ is controlled by a locus at the *K* end of the $H\text{-}2^d$ complex, mapping to the *K* or *I-A* region.

Identification of Nonglycosylated H-2 Precursors. Part of the electrophoretic heterogeneity observed in two-dimensional gel patterns of H-2 antigens is the result of varying degrees of glycosylation of individual polypeptide chains (9, 16). To determine whether H-2K′ is a unique gene product or a differentially glycosylated form of the previously described H-2K^d histocompatibility antigen, the nonglycosylated forms of both H-2K and H-2K′ were identified by radiolabeling B10.D2 spleen cells in the presence of the antibiotic tunicamycin which inhibits the addition of N-linked oligosaccharide units to glycoproteins (23, 24). Fig. 2 *d* and *g* show the gel patterns obtained from normal B10.D2 extracts precipitated by the 34-1-2 monoclonal antibody reactive with H-2K^d, H-2K′^{d}, and H-2D^d or by second monoclonal antibody, 20-8-4, which recognizes H-2K^d and H-2K′^{d} but not H-2D^d.

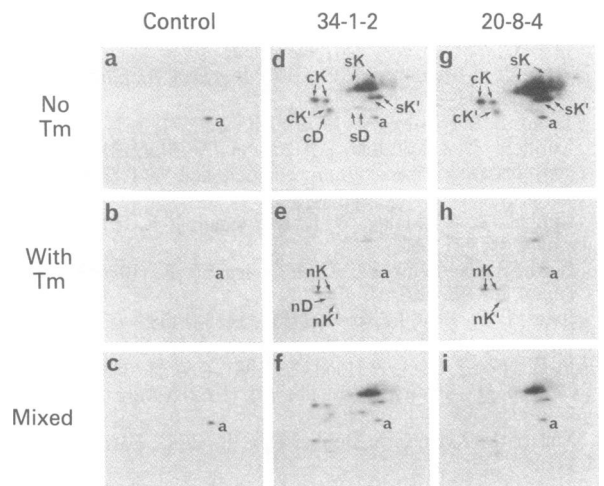

FIG. 2. Identification of nonglycosylated H-2 precursors. (*b*, *e*, and *h*) B10.D2 splenic lymphocytes were treated with tunicamycin (Tm) at 15 μg/ml for 1 hr prior to the addition of [^{35}S]methionine. (*a*, *d*, and *g*) Untreated B10.D2 spleen cell controls. The following monoclonal antibodies were used for immunoprecipitation: control monoclonal MK-S4, anti-I-A^s (*a–c*); monoclonal 34-1-2, anti-H-2K^d,D^d (*d–f*); and monoclonal 20-8-4, anti-H-2K^d (*g–i*). (*c*, *f*, and *i*) Portions of the immunoprecipitates from untreated and tunicamycin-treated cells were mixed and electrophoresed. The nonglycosylated precursor polypeptide (nK, nK′, nD) of each H-2 antigen is labeled in addition to the cytoplasmic (c) and cell surface (s) forms.

The immunoprecipitates from the tunicamycin-treated B10.D2 splenocytes are shown in Fig. 2 *e* and *h*. The nonglycosylated precursor forms of the H-2 antigens (nK, nK′, nD) are clearly evident. Fluorograms of gels of mixtures of the immunoprecipitates from the untreated and tunicamycin-treated cells (Fig. 2 *f* and *i*) show that the spots corresponding to the nonglycosylated polypeptides appeared directly beneath the previously identified cytoplasmic precursor forms, as has been observed for both H-2 and Ia antigens (20). Although tunicamycin does not block the addition of O-linked oligosaccharides, it is unlikely that the difference in size between nK and nK′ is due to O-linked sugars. The addition of such sugars should not cause H-2K to become more basic in its charge. These results therefore suggest that both H-2K and H-2K′ exist as unique, nonglycosylated polypeptides.

It is evident from the gel patterns shown in Fig. 2 that the decrease in apparent molecular weight from the cytoplasmic forms to the nonglycosylated forms seen after tunicamycin treatment is greater for H-2K and H-2K′ than for H-2D. This difference is probably due to the number of N-linked oligosaccharide side chains present. As determined by electrophoresis on one-dimensional NaDodSO$_4$ slab gels with molecular weight markers (not shown), the approximate molecular weight differences from cK and cK′ to nK and nK′ are 8,000, whereas the difference from cD to nD is 5,000. Assuming an approximate molecular weight of 2,500 for each N-linked oligosaccharide core (20, 25), these findings are consistent with three oligosaccharide chains for H-2K^d, in agreement with published results of Kimball *et al.* (26) and two oligosaccharide groups for H-2D^d, as has been reported by Nairn *et al.* (27). Like H-2K^d, H-2K′^{d} appears to possess three oligosaccharide chains.

A final point to be made from Fig. 2 concerns the presence of two distinct nK spots, of the same size but differing in charge, even after tunicamycin treatment. The presence of a second nK spot may be due to charge modification of the H-2K protein, but it is also possible that it represents a third gene product controlled by the *K* end of the $H\text{-}2^d$ complex.

Absence of H-2K′ on Adherent PEC. Because H-2K′ appears to be a distinct protein controlled by the *K* end of the *H-2* complex, we were interested in examining its tissue distribution. To test whether H-2K′ is present on cells other than normal splenic lymphocytes, thioglycollate-induced BALB/c adherent PEC, which consist largely of macrophages, were cultured and their H-2 antigens were analyzed. Cultured adherent PEC stimulated by supernates of Con A-activated spleen cells to increase H-2 antigen expression (18) were also examined. Both the uninduced (u) and Con A supernate-induced (i) PEC expressed the previously recognized H-2K^d and H-2D^d antigens (Fig. 3 *c* and *d*), although the spot patterns are somewhat different from those seen with spleen cells (Figs. 1 and 2). Comparison with the 34-1-2 immunoprecipitates in Figs. 1*g* and 2*d* shows that the H-2K′ antigen is not detectable in the PEC. Preliminary studies have also suggested that the H-2K′ protein also is not expressed by the BALB/c-derived macrophage cell line WEHI-3 cultured either in the absence or presence of Con A supernates (18).

DISCUSSION

Previous serological and biochemical studies have shown that certain haplotypes control multiple antigenically distinct class I molecules (H-2K, D, L, R, Qa-1, Qa-2, and TL) (4, 8–12, 18). Analysis of mouse genomic DNA has revealed the existence of 30–40 class I genes (7), which suggests that there may be additional class I antigens not yet identified. In this investigation, we have shown that two monoclonal antibodies, 34-1-2 and 20-

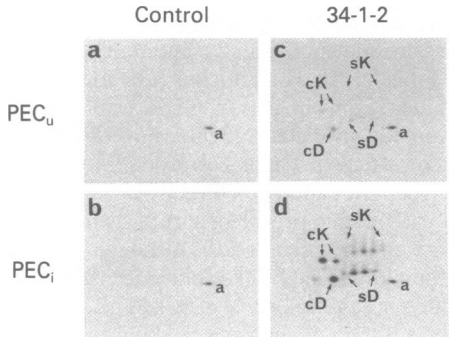

FIG. 3. Two-dimensional polyacrylamide gel electrophoresis of thioglycollate-induced adherent PEC. Cultured PEC were treated either with supernatant fluids from Con A-stimulated mouse spleen cells to induce increased H-2 expression (PEC_i) or with RPMI-1640 medium plus Con A as the uninduced control (PEC_u). Cells were then labeled with [^{35}S]methionine and immunoprecipitated with a control monoclonal antibody (10-3.6, anti-I-A^k) (*a* and *c*) or with an anti-H-2K^d,D^d monoclonal antibody (34-1-2) (*b* and *d*). The cytoplasmic precursor (cK, cD) and cell surface (sK, sD) forms of the H-2 antigens are labeled.

8-4, reactive with class I molecules of the *d* haplotype can detect the presence of a *K* end-controlled antigen, H-2K′, which is distinct from the previously defined H-2K^d protein. H-2K′ has also been immunoprecipitated by using a BALB.B anti-BALB/c (anti-H-2^d) antiserum (unpublished results) and has been seen in BALB/c (*H-2^d*), DBA/2 (*H-2^d*), and D2.GD (*H-2^{g2}*) mice (16, 28). Although H-2K is always seen in the two-dimensional gel patterns, the intensity of the H-2K′ spots has been more variable, suggesting that the titer of antibodies reactive with H-2K′ may vary among alloantisera. These findings are consistent with serological studies described by Démant and Ivanyi in which the presence of two K^d-region molecules, denoted H-2K1^d and H-2K2^d, has been detected (11, 12). At present, the relationship between these molecules and H-2K′ is not known but would be clarified by using, for immunoprecipitation and serological analyses, antibodies which uniquely recognize H-2K′. Thus far, however, no antibodies have been found that uniquely recognize H-2K or H-2K′.

The finding that H-2K and H-2K′ have distinct nonglycosylated precursors suggests that the two proteins differ at the primary structural level. The presence of the H-2K′ protein can be explained by the existence of distinct loci in the K^d region that encode the H-2K and H-2K′ products. However, it is also possible that H-2K′ is the product of translation of a mRNA generated by an alternative mode of processing of the initial H-2K RNA transcript. The apparent generation of multiple mRNAs from individual class I genes has been reported recently by several groups (29–32). Such RNAs could lead to the production of proteins with distinct biochemical, serological, and possibly functional properties.

The absence of H-2K′ on peritoneal macrophages is of particular interest. H-2K′ might be the product of a gene expressed in lymphocytes but not macrophages or the result of tissue-specific RNA processing mechanisms. Whether selectively expressed class I antigens function as tissue-specific restriction elements for T-cell recognition in a manner similar to that of the classical histocompatibility antigens or have some as yet undefined function remains to be established.

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