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Dendritic Cells Permit Identification of Genes Encoding MHC Class II–Restricted Epitopes of Transplantation Antigens

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Summary

Minor or histocompatibility (H) antigens are recognized by CD4$^+$ and CD8$^+$ T lymphocytes as short polymorphic peptides associated with MHC molecules. They are the targets of graft versus host and graft versus leukemia responses following bone marrow transplantation between HLA-identical siblings. Several genes encoding class I–restricted minor H epitopes have been identified, but approaches used for these have proved difficult to adapt for cloning class II–restricted minor H genes. We have combined the unique antigen-presenting properties of dendritic cells and high levels of episomal expression following transfection of COS cells to identify a Y chromosome gene encoding two HY peptide epitopes, HYA^b and HYE^k.

Introduction

Histocompatibility (H) antigens are the products of polymorphic genes recognized by the immune system following the exchange of organs or tissues between genetically dissimilar individuals. The MHC molecules themselves (HLA in humans, H2 in mice) are the most polymorphic, and exchange of tissues between individuals expressing different MHC alleles causes rapid graft rejection involving CD4$^+$ and CD8$^+$ lymphocytes and antibodies (Ting and Simpson, 1989). However, a vigorous T cell response is still mounted even when donor and recipient are MHC matched. This response is directed against minor H antigens. These are peptide products of ubiquitously expressed intracellular proteins that demonstrate a more limited, mostly di-allelic, polymorphism. The peptides enter the biosynthetic pathway of MHC molecules and are expressed at the cell surface within the peptide binding groove of the MHC molecules (Bjorkman et al., 1987a, 1987b), where they can be recognized by circulating CD4$^+$ and CD8$^+$ T cells (Roopenian, 1992).

Responses to minor H disparities between MHC-matched donor/recipient pairs are restricted to T cells, since the target antigens are self-MHC peptide complexes not readily recognized by antibodies (Simpson and Roopenian, 1997). Such minor H–specific T cells can be isolated in vitro in the presence of antigen-presenting cells (APC) expressing the relevant antigens, cloned, and maintained in long-term culture (Tomonari, 1983; Simpson and Chandler, 1997). To date, the major focus has been on CD8$^+$ clones to identify several class I–restricted minor H epitopes. This is in part on the assumption that CD8$^+$ cytotoxic cells are the main effector cells in graft rejection but also because class I–restricted epitopes have been relatively easy to express both in transfectants (Scott et al., 1995; Mendoza et al., 1997) and by peptide pulsing of APC (Rotzschke et al., 1990; den-Haan et al., 1995). However, to understand the induction phase of the response, when CD4$^+$ "help" is required to optimize generation of CD8$^+$ cells (Roopenian, 1992) and since CD4$^+$ cells themselves can be effectors (Hung et al., 1998), we have focused on ways of identifying the MHC class II–restricted epitopes recognized by CD4$^+$ cells.

There are significant differences in the way in which MHC class I and class II molecules become loaded with peptides for presentation to T cells. Peptides from endogenously produced proteins are loaded into newly synthesized class I molecules after translocation from the cytoplasm into the endoplasmic reticulum (ER) via the ATP-dependent peptide transporter (TAP) molecules (Neefjes et al., 1993). In contrast, the peptide loading of class II molecules occurs in the endocytotic compartments (MIIC). Here, invariant chain (Ii) molecules associated with class II molecules during biosynthesis are enzymically removed and replaced by peptides (Cella et al., 1997). These can be derived either from exogenous proteins after endocytosis (Malcherek et al., 1998) or from endogenously produced proteins in the MIIC (Weiss and Bogen, 1991; Sanderson et al., 1995). The distinction between class II–associated self-peptides loaded endogenously and those exogenously derived is important and will influence the method chosen to identify class II–restricted epitopes of cell-autonomous origin, such as minor H antigens and tumor antigens.

Efforts to transfect expression constructs containing candidate minor H antigen genes into recipient APC bearing the relevant MHC class II molecules have failed to lead to detectable expression of class II–restricted epitopes (data not shown). In model systems where genes or smaller fragments encoding peptide epitopes

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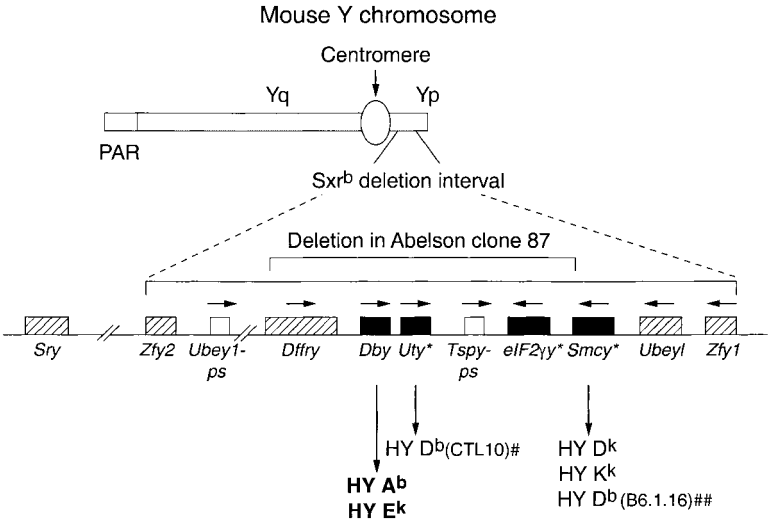

Figure 1. Schematic Representation of the Mouse Y Chromosome

This shows the location of the Sxr^b deletion interval on the short arm, Yp, mouse Y chromosome, and a transcription map of Sxr^b. The genes are represented by boxes: open for nonfunctional genes, hatched for testis-specific, and black for ubiquitously expressed genes. Their orientation is shown by the arrows pointing toward the 3′ end. The asterisks indicate genes whose X chromosome homologs are known to escape X inactivation. Above is shown the extent of the deletion in Abelson clone 87. The HY epitopes expressed by the genes are given below. Single pound sign, HYD^b epitope recognized by clone CTL10 (Greenfield et al., 1996); double pound sign, HYD^b epitope recognized by clone B6.2.16 (Markiewicz et al., 1998). The two epitopes in bold are described in this paper.

from, for example, influenza haemagglutinin (HA), ovalbumen (OVA), hen egg lysozyme (HEL), or moth cytochrome C (MCC) were transfected into APC, Ii was necessary for optimal cell surface expression. Depending on the experimental system, Ii was required either as a cotransfection partner or as a fusion construct, using the whole Ii molecule (for peptides) or its N-terminal lysosomal targeting sequence for loading peptides into class II molecules expressed at the cell surface for subsequent T cell recognition (Sanderson et al., 1995; Nakano et al., 1997).

In this paper, we report results of experiments in which constructs containing candidate Y chromosome genes, with or without Ii sequences, have been transfected into COS cells. We also describe the additional antigen-processing step, using syngeneic female bone marrow (BM)–derived dendritic cells, found necessary to obtain expression of the MHC/HY peptide complexes for T cell recognition. These results imply that the expression of at least some minor H epitopes restricted by MHC class II molecules depends on reprocessing by professional antigen-presenting cells. In vivo, this implies an important role for indirect presentation in generating CD4$^+$ helper and effector T cells for rejection of transplants between MHC-matched individuals (Bevan, 1976).

Results

Identification of Candidate Genes

All the genes that encode HY epitopes have been mapped to the Sxr^b deletion interval on the short arm of the mouse Y chromosome between *Zfy1* and *Zfy2* (Simpson and Page, 1991; McLaren et al., 1984) (Figure 1). A panel of immunoselected radiation mutant clones with small deletions within this region showed that several distinct genes were likely to encode different HY epitopes. In particular, clone 87, with a deletion extending between and including *eIF2γY* and *Dffry* (M. J. M., unpublished data) does not express the HYD^b and HYA^b epitopes (King et al., 1994). Within Sxr^b, several genes exhibit testis-specific expression, two are nonfunctional, while four, *Smcy*, *Uty*, *Dby*, and *eIF2γY*, are ubiquitously expressed (Greenfield et al., 1996; Ehrmann et al., 1998; Mazeyrat et al., 1998; Agulnik et al., 1999) (Figure 1) and have X chromosome homologs that for the three genes that have been examined escape X inactivation (Ehrmann et al., 1998; Greenfield et al., 1998; Agulnik et al., 1999). The mouse gene *Smcy* encodes three HY epitopes (HYK^k, HYD^k, and HYD^b) (Scott et al., 1995; Simpson et al., 1997; Markiewicz et al., 1998), while *Uty* encodes the immunodominant HYD^b epitope (Greenfield et al., 1996). The human homolog *SMCY* encodes the HLA-B7- (Wang et al., 1995) and -A2 (Meadows et al., 1997)-restricted HY epitopes and *UTY* the -B8 epitope (Warren et al., 1999). In attempts to identify candidate genes for additional HY epitopes restricted by MHC class II molecules, we have focused on *Smcy*, *Uty*, *Dby*, and *Eif2γy*.

Establishing a Protocol to Enable Detection of an H2A^b-Restricted Peptide Epitope

COS cells were transiently transfected with a construct containing the complete HEL sequence in pcDNA1. This was transfected alone and cotransfected with Ii or as a fusion construct comprising the first 80 amino acids of Ii and HEL (I80-HEL) (Sanderson et al., 1995) (Figure 2). These HEL constructs were transfected with or without the α and β chains of H2A^b, also in pcDNA1. Figure 3A shows the results of testing the cells 48 hr after transfection for their ability to stimulate the H2A^b/HEL-specific hybridoma B04H9.1. As expected, in the absence of cotransfected H2A$^b\alpha\beta$, none of the transfectants were stimulatory. The I80-HEL COS + $\alpha\beta$ transfectants stimulated a good response directly, but COS + $\alpha\beta$ transfected with HEL or Ii + HEL stimulated poorly or not at all (Figure 3A). The addition of day 6 bone marrow–derived dendritic cells (DCs) from syngeneic female mice to the transfectants at 48 hr, followed by a further 24 hr incubation to allow reprocessing of the antigen expressed by the COS cell transfectants, resulted in even low numbers of the HEL COS + $\alpha\beta$ transfectants stimulating the hybridoma (Figure 3B). Addition of DCs did not significantly increase the level of presentation by the I80-HEL COS + $\alpha\beta$ transfectants. Interestingly, in the absence

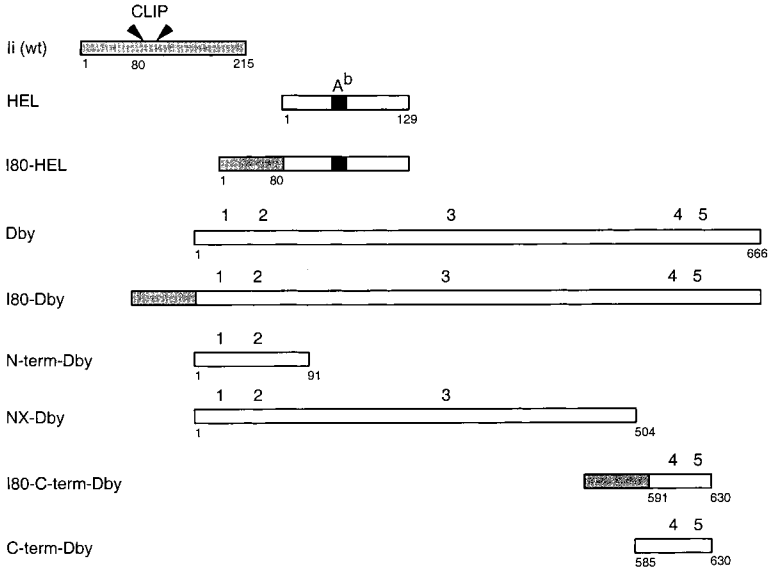

Figure 2. Schematic Representation of Expression Constructs of HEL and *Dby*

The wild-type (wt) murine Ii, p31 cDNA encodes a type II transmembrane protein of 215 residues. The region containing the class II–associated Ii peptide (CLIP) is indicated. I80 constitutes the first 80 amino acids of Ii containing an endosomal targeting sequence. This fragment has been used to create fusion proteins with HEL, *Dby*, and C-terminal *Dby*. The HEL protein contains an A^b MHC class II–restricted epitope at residues 74–91, indicated by a shaded box. cDNAs encoding full-length and truncated *Dby* fragments were generated (see Experimental Procedures), and the presence or absence of five potential $H2A^b$ binding motifs are indicated above each construct, while the amino acid residues included in each construct are shown below.

of cotransfected $\alpha\beta$ chains, none of the HEL constructs stimulated the hybridoma, even in the presence of BM DCs. This suggests a crucial role for $H2A^b$ $\alpha\beta$ in the protection of HEL peptides.

Identification of the Gene Expressing MHC Class II–Restricted HY Epitopes

Having established that reprocessing of HEL expressed by COS + $\alpha\beta$ transfectants by BM DCs resulted in much higher expression of the $H2A^b$/HEL epitope, the candidate mouse Y chromosome genes were transfected into COS cells, allowed to transiently express for 48 hr followed by addition of female 6d BM DCs ($H2^b$ or $H2^k$) for a further 24 hr. Figure 4 shows the results of screening the transfectants for HY expression: Figure 4A, IL-2 production by the HYA^b-specific hybridoma and Figure 4B, IFNγ production by the HYE^k-specific clone. Clearly, *Dby* expresses both MHC class–II restricted A^b and E^k HY epitopes. No stimulatory activity was detected in cells transfected with *Smcy*, *Uty*, or *Eif2*γ*y*.

Requirements for Optimal Expression of a "Natural" Endogenously Expressed Class II–Restricted Minor H Antigen

COS cells were transfected with *Dby* in the presence and absence of Ii and as an I80-fusion construct, with and without $H2A^b$ α and β and with and without added BM DCs. In the absence of dendritic cells, only the I80-*Dby* COS + $\alpha\beta$ transfectants stimulated the HYA^b-specific T cell hybridoma (Figure 5A). In contrast, with BM DCs, maximal stimulation was given by COS cells transfected with *Dby* alone (Figure 5B). Cotransfection of Ii gave a slight reduction in the ability of the COS-*Dby* transfectants to stimulate the hybridoma and, in contrast to the experiments using HEL as model antigen, cotransfection of the $H2A^b\alpha\beta$ chains appeared to further inhibit rather than enhance expression of the HYA^b epitope by the transfected cells. This may reflect quantitative differences in levels of *Dby* expression when several genes are cotransfected simultaneously. These results show naturally expressed MHC class II–restricted epitopes do not require $H2A^b$ molecules to stabilize their

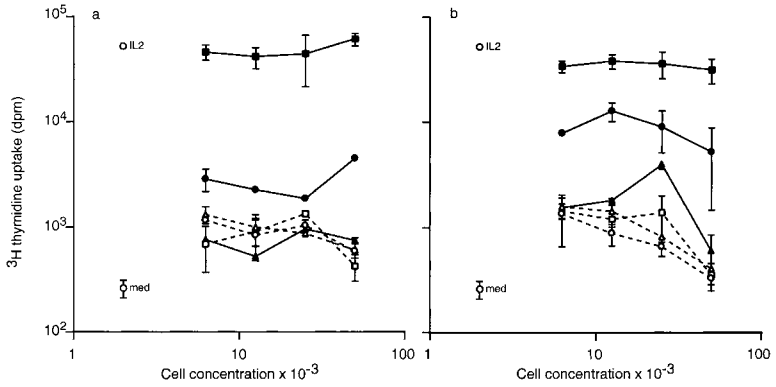

Figure 3. Role of $H2A^b$ α and β Chains and Reprocessing by Female BM DCs in the Presentation of the $H2A^b$-HEL Epitope

IL-2 production by the HEL A^b hybridoma stimulated by COS cell transfectants in the absence (A) or presence (B) of d6 female BM DCs.

(A) COS cells transfected with HEL (open circles, dotted line); HEL + Ii (open triangles); I80-HEL (open boxes); HEL + $\alpha\beta$ (closed circles); HEL + Ii + $\alpha\beta$ (closed triangles); and I80-HEL + $\alpha\beta$ (closed boxes).

(B) d6 female BM DCs plus COS cells transfected as in (A). The results represent the mean and SD of triplicate wells for each dilution of transfectant and are expressed as dpm of an IL-2-responsive T cell clone. Control responses of the T cell clone to IL-2 and medium alone are shown (open circles).

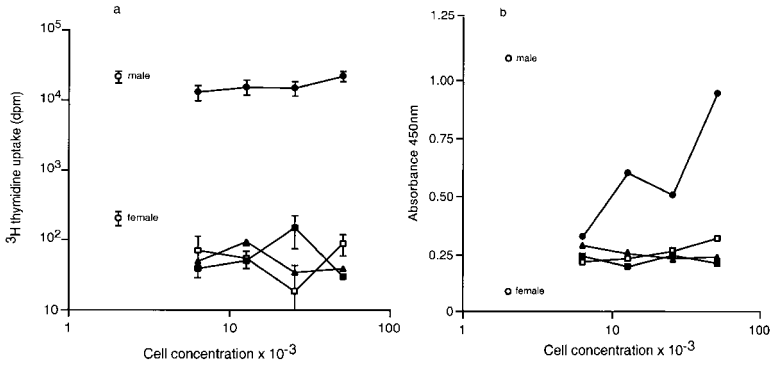

Figure 4. Stimulation of HYA^b-Specific Hybridoma and HYE^k-Specific T Cell Clone by COS Cells Transfected with Candidate Mouse Y Chromosome Genes

(A) IL-2 production by the HYA^b hybridoma stimulated by COS cells transfected with *Dby* (closed circles); *Smcy* (closed boxes); *Uty* (open sqaures); and *eIF2γY* (closed triangles). The transfectants were assayed in the presence of d6 C57BL/6 female BM DCs. Hybridoma stimulation by control male and female C57BL/6 splenocytes is indicated by open circles. The results represent the mean and SD of triplicate wells for each dilution and are expressed as dpm of an IL-2-responsive T cell clone.

(B) IFNγ production by the HYE^k T cell clone stimulated by COS cells transfected with *Dby* (closed circles); *Smcy* (closed boxes); *Uty* (open boxes); *eIF2γY* (closed triangles). The transfectants were assayed in the presence of d6 CBA female BM DCs. Clone stimulation by control male and female CBA splenocytes and medium alone is indicated by open circles. Results of screening of pooled supernatants by ELISA are expressed as absorbance at 450 nm.

intracellular processing. The I80-*Dby* + αβ fusion constructs gave similar stimulation in the presence or absence of BM DCs.

Identification and Properties of the HYA^b Peptide Epitope

To locate the HYA^b epitope, pcDNA1 constructs comprising the first 91 amino acids residues (N-terminal), residues1–504 (N + X), and the C-terminal residues (591–630) of *Dby* (Figure 2) were transfected into COS cells, reprocessed by female BM DCs, and screened for HY expression. The C-terminal fragment expresses the HYA^b epitope (results not shown). Comparison of the sequences of *Dby* with *Dbx* indicated five peptides with potential H2A^b binding motifs (Wall et al., 1994) in which there is at least one amino acid difference between the X and the Y copy of the gene. These were synthesized and tested with the HYA^b clone. The results (Figure 6A) show that only peptide 5 (p5-*Dby*-NAGFNSNRANSSRSS) stimulated and at nanomolar concentrations. This is therefore the cognate peptide defining the HYA^b epitope. The *Dbx* homolog of peptide 5 (p5-*Dbx*-SSSFSSSRASS SRSG) did not stimulate (Figure 6A). The binding of p5-*Dby* and its *Dbx* homolog p5-*Dbx* to H2A^b was tested in a competition assay using biotinylated CLIP as indicator peptide. As shown in Figure 6B, p5-*Dby* and p5-*Dbx* peptides bind equally to A^b (IC50 12 μM), implying that both have the potential to be presented by H2A^b molecules. In addition, although nonstimulatory to our T cell clone and hybridoma, peptide 4 (p4-*Dby*) showed strong binding to H2A^b molecules (IC50 = 19 μM). The other peptides demonstrated moderate, weak, or no binding. Substitution of serine (S) for asparagine (N) at position 4 (assuming phenylalanine (F) in position 1 of the "core" peptide that occupies the MHC binding groove) resulted in complete loss of T cell stimulatory activity (Figure 6C). Substitution of serine for asparagine at position 7 gave negligible loss of activity.

Identification of the HYE^k Epitope

To locate the HYE^k epitope, nine synthetic peptides expressing potential H2E^k binding motifs (Fremont et al., 1996) and showing sequence differences between the *Dbx* and *Dby* homologs were tested for their ability to stimulate the HYE^k clone. Figure 7 indicates that *Dby* peptide 8, REEALHQFRSGRKPI, is the cognate peptide, giving maximal stimulation at a concentration of 100 nM but causing a significant response at 1 nM. The *Dby* peptide 8 equivalent derived from the *Dbx* sequence, REEALHQFRSGKSPI, gave only a low level of stimulation of the HYE^k-specific clone at concentrations of 100μM, as did *Dby* peptide 7 (Figure 7).

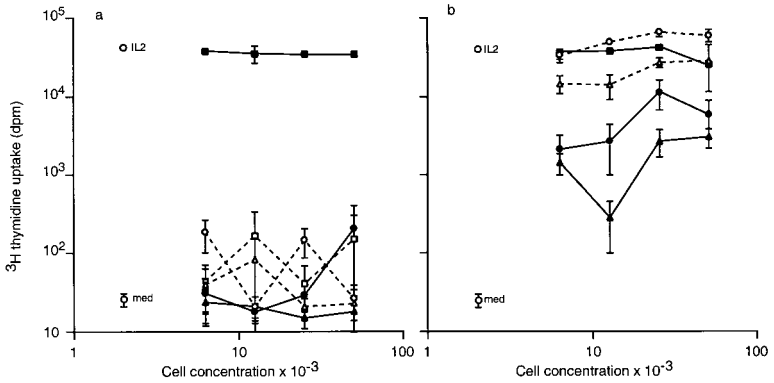

Figure 5. Role of H2A^b α and β Chains and Reprocessing by Female BM DCs in the Presentation of the HYA^b Epitope

IL-2 production by the HYA^b hybridoma stimulated by COS cell transfectants in the absence (A) or presence (B) of d6 female BM DCs.

(A) COS cells transfected with *Dby* (open circles); *Dby* + Ii (open triangles); I80-*Dby* (open boxes); *Dby* + αβ (closed circles); *Dby* + Ii + αβ (closed triangles); and I80-*Dby* + αβ (closed boxes).

(B) d6 female BM DCs plus COS cells transfected as in (A). The results represent the mean and SD of triplicate wells, as in Figure 3.

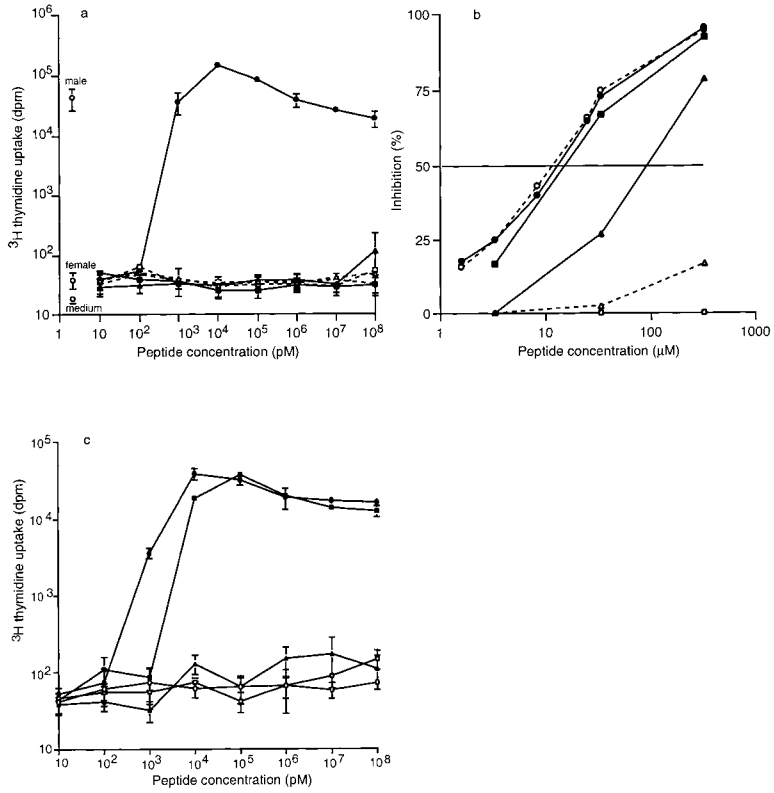

Figure 6. Identification of the Peptide Epitope for HYA^b and Demonstration that the *Dbx* Homolog of the HYA^b Peptide Binds to H2A^b with the Same Affinity as the *Dby* Peptide

(A) Stimulation of HYA^b clone B9 by candidate *Dby* peptides. Peptide 1, p1*Dby* (open triangles, dotted line): DQQFVGLDKSSDNQ; peptide 2, p2*Dby* (closed triangles): DKDAYSSFGS RDSRGK; peptide 3, p3*Dby* (open boxes): RPCVVYGGADTVQQIRD; peptide 4, p4*Dby* (closed boxes): ARDYRQSSGSANAG; peptide 5, p5*Dby* (closed circles): NAGFNSNRANS SRSS; *Dbx* peptide 5 p5*Dbx* (open circles, dotted line): SSSFSSSRASSSRSG. Serial 10-fold dilutions of peptide were tested. The results give the mean and SD of triplicate wells and are expressed as dpm. Control responses of the clone to male and female splenocytes and to medium alone are shown (open circles).

(B) The peptides representing HYA^b epitope (p5*Dby*), its *Dbx* homolog (p5*Dbx*), and the four *Dby* peptides containing the H2A^b binding motif *Dby* (p1-p4) as described above were tested for inhibition of binding of 0.8 μM biotinylated CLIP peptide to purified H2A^b molecules. The values represent the means of triplicate wells from a single experiment, which was repeated twice. The symbols used are as described in (A).

(C) Stimulation of HYA^b clone by substituted Dby peptides. p5*Dby* (closed circles): (NAGF NSNRANSSRSS); p5*Dbx* (open circles): (SSS FSSSRASSSRSG); p5-S4 (open boxes): (NAG FNSSRANSSRSS); P5-S7 (closed boxes): (NAG FNSNRASSSRSS); p5-S4, S7 (closed triangles): (NAGFNSSRASSSRSS). Serial 10-fold dilutions of peptide were tested as in (A).

Discussion

The results presented here describe the MHC class II–restricted minor H gene as *Dby*. This gene expresses two HY determinants, one recognized in association with H2A^b, the other with H2E^k. *Smcy* and *Uty* have been shown to express all the MHC class I–restricted HY peptide epitopes identified to date (Figure 1 and Simpson et al., 1997). Although they might, in principle, also encode those restricted by class II molecules, positional mapping of HY, H3, and H13 loci indicate that each locus is comprised of a gene complex containing at least two genes, separable by recombination or mutation (Roopenian, 1992; King et al., 1994; Roopenian, 1999). Our finding that the gene encoding the MHC class II–restricted epitopes, *Dby*, is separate from but closely linked to the *Smcy* and *Uty* genes provides direct evidence that this is the case.

The specific cellular functions of proteins encoded by the minor H genes are largely unknown. They are heterogeneous: *Smcy* shares homology with the retinoblastoma binding protein, RBP2 (Scott et al., 1995), and *Uty* belongs to a family of tetratrico repeat proteins (Greenfield et al., 1996), H3, H13, and H60 are all novel genes (Mendoza et al., 1997; Malarkannan et al., 1998; Zuberi et al., 1998; Roopenian, 1999). They are ubiquitously transcribed. *Dby* belongs to a well-conserved family of genes coding for known or putative RNA helicases. This family of so-called DEAD box proteins shares several core structural elements, including an Asp-Glu-Ala-Asp (DEAD in single letter code) motif, and are involved in modulation of secondary and tertiary RNA structure (Fuller-Pace, 1994). They have roles in a range of cellular processes, including splicing, ribosomal assembly, and translation, and may be localized to the nucleus or cytoplasm. DEAD box proteins fall into two subgroups (Gee and Conboy, 1994). One, including *Dbx* (mDEAD3) and *Dby* (mDEAD2), comprises proteins similar to the autosomally encoded mouse testis-specific PL10 gene (Leroy et al., 1989) and shows extensive homology around each of six small conserved motifs within the central core region and a less conserved C-terminal domain, which is rich in Arg, Ser, Gly, and Phe. The HYA^b epitope lies within this distinct C-terminal region of *Dby*. The other group comprises translation initiation factors including mouse *eIF-4AI* and *eIF-4AII*, and mDEAD5, which all contain a novel peptide motif of 19 amino acids.

The HYA^b peptide epitope defined here as NAGFNSN RANSSRSS has a single aromatic residue, phenylalanine, that is, according to the H2A^b binding motif, the likely primary anchor residue, P1 (Wall et al., 1994). As the binding groove of MHC class II molecules typically accommodates peptides of nine residues in length (Stern et al., 1994), the likely "core" sequence of the HYA^b is FNSNRANSS. Notably, the sequence encoded by the X homolog *Dbx*, FSSSRASSS, contains the same primary anchor residue and two other putative H2A^b

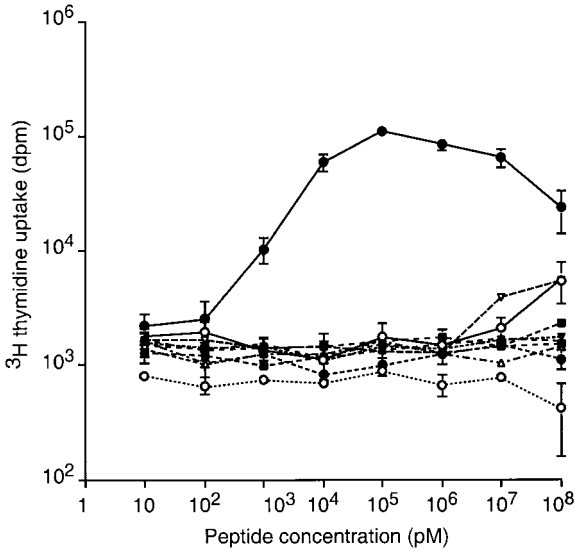

Figure 7. Identification of the Peptide Epitope for HYE^k

Stimulation of HYE^k T cell clone by *Dby* peptides. Peptide 1 (closed circles, dashed line): DNQNGGGNTESKGRY; peptide 2 (open circles, dotted line): SFGSRDSRGKPNYFSDRGS; peptide 3 (open boxes): GRNDYDGIGGRDRTG; peptide 4 (closed boxes): GEALKAMKEN GRYGR; peptide 5 (closed triangles, pointing up): YGGADTVQQIRDL ERG; peptide 6 (open triangles, pointing up): TQKVVWVEELDKRS; peptide 7 (open triangles, pointing down): DSLENFLFQERYA; peptide 8 (closed circles, solid line): REEALHQFRSGRKPI; peptide 9 (closed triangles, pointing down): GSANAGFNSNRANSSRSS; *Dbx* peptide (open circles, solid line): REEALHQFRSGKSPI. Serial 10-fold dilutions of peptide were tested for their ability to stimulate the HYE^k T cell clone in the presence if 1 IU/ml recombinant IL-2. The results give the mean and SD of triplicate wells and are expressed as dpm.

contact residues, alanine at P6 and serine at P9: this could explain very similar affinities of both peptides for H2A^b. Sequence differences between the *Dby* and *Dbx* peptides are at positions P2, P4, and P7, each changing from asparagine to serine. Positions P4 and P7 are described as T cell receptor (TCR) contact residues in several models (Sette et al., 1987; Daniel et al., 1998); (Garboczi et al., 1996; Garcia et al., 1998). We have found by substituting serine for asparagine at positions P4 and P7 that N at P4 is crucial for T cell recognition. P7 appears less critical since substitution of S for N here had little effect on peptide recognition. In the case of the HYE^k epitope, REEALHQFRSGRKPI, P1 and P4 are generally noncharged, P6 often glutamine, asparagine, or alanine, and P9 is almost always lysine or arginine. The core peptide is therefore likely to be LHQFRSGRK. Comparison of the equivalent sequence from *Dbx*, LHQFRSGKS, indicates only two amino acid differences, a conservative lysine for arginine at position P8 and serine for lysine at P9. This latter substitution is likely to affect the binding of the *Dbx* peptide since the P9 pocket comprises of a long hydrophobic tunnel with glutamic acid at the base and is unlikely to accommodate the serine residue (Fremont et al., 1996). The finding that the *Dbx* peptide gives very low levels of stimulation at high concentrations (100 μM) is consistent with this interpretation.

Our finding that the peptides giving rise to expression of MHC class II–restricted minor transplantation antigens, like their class I–restricted counterparts, are the result of allelic variation, in this case between X and Y homologs, of the same gene products, which is in contrast to the recent findings for class II–restricted tumor antigens. For example, one is derived from the protein product of a mutated gene, CDC27, and does not provide the *neo*-epitope. Rather, it changes the cellular localization of the protein, allowing antigenic peptide fragments derived from CDC27 to be loaded into class II molecules (Wang et al., 1999a). Another type of unique class II–restricted tumor epitope is a novel peptide resulting from a fusion gene between the low density lipid receptor (LDLR) and a fucosyl transferase gene (FUT) transcribed in an antisense direction following a tumor-specific chromosomal translocation (Wang et al., 1999b). However, in these experiments, it is not altogether clear whether the protein generated following transfection is presented by the transfectant itself or is taken up by and represented by T cells. A third recently described tumor antigen is a *neo*-epitope created by mutation in a glycolytic enzyme, TPI (Pieper et al., 1999). This mutant TPI peptide can sensitize target cells for recognition by class II–restricted tumor-specific CD4$^+$ T cells at nanomolar concentrations, in contrast to the nonmutated melanoma-associated tyrosinase epitopes identified in several tumors, which require micromolar amounts of peptide in detection assays (Topalian et al., 1996; Pieper et al., 1999). The HYA^b and HYE^k peptide epitopes from *Dby* are also active at nanomolar concentrations (Figures 6 and 7), and this may be characteristic of the highly sensitive recognition by T cells specific for this type of transplantation antigen.

Minor H antigens are in general ubiquitously expressed on normal cells as well as some leukemia cells (den-Haan et al., 1998; Warren et al., 1999). Their display at the cell surface requires that peptide fragments derived from proteins in various cellular compartments intersect with the MHC biosynthetic pathways. MHC class I binding peptides follow a well-characterized TAP-dependent route before loading into class I molecules, and MHC/minor H peptide complex expression at the cell surface is readily reconstituted in target cells transfected with genomic or cDNA fragments (De-Plaen et al., 1988; Boon et al., 1994; Mendoza et al., 1997). This is not the case for class II loading of endogenous peptides, and targeting the peptide products to the right cellular compartment has presented a major problem, certainly using model antigens. To overcome this, invariant chain constructs have been incorporated, where APC other than dendritic cells or B cells are used, to load peptides into class II molecules for T cell recognition. Although sometimes cotransfection of the whole Ii molecule is found to enhance expression of endogenously expressed transfected genes, on occasion the presence of Ii inhibits antigen expression (Peterson and Miller, 1990; Long et al., 1994; Armstrong et al., 1997; Zhong et al., 1997). More recently CLIP-substituted invariant chain has been used to facilitate peptide loading into MHC class II molecules (van-Bergen et al., 1997; Fujii et al., 1998; Malcherek et al., 1998). However, the most efficient way of targeting endogenously expressed genes into the MIIC compartments has been by making

fusion genes with the N-terminal 80 amino acids of invariant chain (Sanderson et al., 1995). In the experiments reported here, cotransfection of Ii did not enhance antigen presentation, but the fusion constructs I80-HEL and I80-*Dby* both gave high levels of expression when transfected into COS cells with H2A α and β. Our unexpected finding that cotransfection of the H2A$^b\alpha\beta$ chains was an absolute requirement for the HEL-A^b peptide expression even in the presence of dendritic cells suggests that the MHC class II α and β chains perform an additional role of stabilizing the HEL-A^b peptide within the cell, thus preventing its further enzymatic cleavage within the cytosol. In contrast, we found that expression of the "natural" endogenously derived HYA^b peptide epitope did not require H2A^b $\alpha\beta$ for expression. Rather, we found that in the absence of I80 to direct *Dby* expression to the relevant endosomal compartment, COS cells were unable to process and express the HYA^b epitope from the transfected *Dby* gene. There was an absolute requirement for DCs to reprocess the antigen. This suggests that, more generally, T cell responses in vivo to this type of antigen will be indirect, following processing of donor antigens by recipient APCs.

The use of dendritic cells as APC may provide an effective alternative route following transfection to optimize expression of candidate genes or cDNA pools containing genes encoding class II–restricted minor H epitopes. It is one which makes no assumptions about the normal pathway taken by the protein precursor before generation and loading of the peptide—it could be endogenous, or follow reprocessing and presentation of secreted material, or that derived from dying cells (Bevan, 1976; Stockinger et al., 1993; Albert et al., 1998a, 1998b). Providing a source of autologous or MHC-matched dendritic cells is available, their use as final APC following transient transfection to provide high levels of protein expression obviates the need to "tailor make" recipient cells by transfection of additional molecules such as DR matched for the restriction molecule and DM to facilitate peptide loading. This technique could therefore be used to identify minor H genes recognized by CD4$^+$ T cells isolated from bone marrow transplant (BMT) patients for whom recipient APCs had been stored before transplantation, as is the practice for chronic myelogenous leukemia (CML) patients given BMT.

In addition to serving as an amplification or enabling step during T cell expression cloning for class II–restricted epitopes of minor H antigens, the special qualities of dendritic cells at different stages of differentiation (Winzler et al., 1997) could be used to present individual minor H peptides in vivo to facilitate induction of tolerance or immunity. From a report of the ability of dendritic cells to process and represent a T cell epitope peptide from a transgenic class II chain, E^a, it is clear that under certain circumstances, tolerogenic signals can be generated, both in vitro and in vivo (Inaba et al., 1998). It is also clear from the work of Waldmann and Cobbold that "infectious" and "bystander" tolerance to minor H antigens can result in the spread of tolerance to one set of minor antigens (albeit not identified at the peptide level in these experiments) to others present on the same APC (Cobbold et al., 1996; Davies et al., 1996). There is a clinical need to manipulate the immune response to minor H antigens following bone marrow transplantation between HLA-identical individuals, to downregulate harmful graft versus host (Goulmy et al., 1996), and to optimize graft versus leukemia responses. It is likely that such immunological manipulation will require knowledge both of class II– as well as class I–restricted minor H epitopes. This paper provides new information about ways to approach identification of the class II–restricted epitopes, with this end in mind.

Experimental Procedures

Plasmid DNA Constructs

cDNAs encoding A$^b\alpha$ and A$^b\beta$, HEL, and I80-HEL cloned into pcDNA1 (Invitrogen) were kindly provided by N. Shastri (Department of Molecular and Cell Biology, University of California, Berkeley). I80-HEL is a fusion construct encoding the first 80 residues of Ii and full-length HEL (129 amino acids). The full-length Ii cDNA, cloned into the EcoRI site of pExV-3 (Miller and Germain, 1986), was obtained from A. Sponaas (Institute for Zoology, University of Bonn). The I80 region of Ii was subcloned as an EcoRI–HindIII fragment into pBK-CMV (Stratagene) for construction of *Dby* fusion constructs. The full-length *Dby* cDNA was cloned into the NotI site of pcDNAI for expression studies. N-terminal *Dby*, encoding the first 91 amino acids of *Dby*, was prepared by subcloning a 5′ BamHI into pcDNA1. NX-*Dby* (encoding amino acids 1–504) was similarly prepared by subcloning a NotI–XbaI fragment into pcDNA1. C-terminal *Dby* constructs were prepared by PCR using the oligonucleotide primers 5′-ACCATGGGACGTTCTAAAAGC-3′ (forward) and 5′-ACGCTCGA GTTATCCTCTGTTGTGGCT-3′ (reverse). For preparation of I80 C-terminal *Dby*, the forward primer was 5′-CGCAAGCTTTTCAGTGGAG GATTT-3′. This was subcloned into pBK-CMVI80 to give the completed fusion construct . To produce full-length I80-*Dby*, a 5′ fragment encoding the first 91 amino acids of *Dby* was generated by PCR with primers 5′-CCGAAGCTTAGTCAAGTGGCAGCG-3′ (forward) and 5′-CCTGGATCCACTTCCACGAT-3′ (reverse). This was subcloned into pcDNA1 and combined with a 3′ BamHI–NotI fragment encoding the remaining 575 residues of the *Dby* C terminus. The complete coding sequence of *Dby* was subcloned into pBK-CMV-I80. All constructs were sequenced to confirm fidelity of their translational reading.

Cells and Transfections

COS1 cells were a kind gift of J. Dunne, ICRF. The HYA^b-specific T cell hybridoma (B9BW) was from a fusion between the B9 clone (Simpson and Tomonari, 1989) and the BWCD4 cell line (derived from Z.8 [Karttunen et al., 1992] after transfection with CD4, a kind gift from N. Glaichenhaus, CNRS Université de Nice). The HYE^k-specific clone, R2.2, derived from transgenic mice expressing the TCR α and β chain from the HYE^k T cell clone, A1 (Zelenika et al., 1998), was provided by E. Adams, University of Oxford. CBA/Ca and C57BL/6 mice were obtained from OLAC, Oxon, UK. Bone marrow dendritic cells were prepared from the femurs and tibias of female donor mice and cultured at 2 to 5 $\times$ 10^5/ml in RPMI 1640 medium supplemented with 5 $\times$ 10^{-5} M 2-mercaptoethanol,10% fetal calf serum, and 5%–10% GMCSF (supernatant from X63-Ag8 cells) for 6 days. DNA (10 μg each construct) was transfected into COS cells (1 to 2 $\times$ 10^6) by electroporation using a BioRad Gene Pulser apparatus as described previously (Scott et al., 1992). The cells were resuspended in DMEM medium and incubated in 6-well plates at 37°C for 48 hr.

Screening Transfectants for HY Epitope Expression

After 48 hr, COS cell transfectants were harvested using a rubber policeman and resuspended at 5 $\times$ 10^5/ml in RPMI 1640 medium. Fifty or one hundred microliter triplicate samples of transfectants were placed in 96-well flat-bottomed plates and 3$\times$ 2-fold dilutions were made. BM DCs (50 μl at 5 $\times$ 10^5/ml) were added to the 50 μl samples and incubated for a further 24 hr. The HY-specific clone (R 2.2) or hybridoma (B9BW) cells were added (1 to 2 $\times$ 10^4/well in 100 μl) to the transfectants ($\pm$ DCs) and incubated for 24 hr for the

hybridoma and 48 hr for the T clone. For the R2.2 clone, 1 IU rIL-2/ml was also added. One hundred microliter supernatant was harvested from each well and assayed for IL-2 or IFNγ respectively. IL-2 production was measured by proliferation using an IL-2-dependent T cell clone. One hundred microliter cells at 10^5/ml were added to the supernatants. The cells were incubated at 37°C for 40 hr and ^{3}H-thymidine was added for the last 12–18 hr of culture. The cells were harvested and counted using a Wallac 1205 β plate counter (Wallac Pharmacia, Milton Keynes, UK). The results are expressed as dpm. IFNγ was measured by ELISA using XMG1.2 anti-mouse IFNγ monoclonal antibody (PharMingen International, Becton Dickinson, UK).

Screening Peptides for HY Epitope Expression

Peptides were synthesized using FMOC-protected amino acids and (2-(1-H-Benzotriazol-2-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU) activation chemistry. After purification by HPLC, the fidelity of synthesis was confirmed by mass spectrometry. Synthetic peptides were made up as 1 mM stocks in PBS and filter sterilized. Serial 10-fold dilutions were made in triplicate. Irradiated (30 gy) female splenocytes (5×10^5/well) were added to each well. For HYA^b, the T cell clone B9 (2×10^4/well) was added, and the cells were incubated for 72 hr. ^{3}H-thymidine was added for the last 12–18 hr culture, and the cells harvested and counted as described above. For HYE^k the R2.2 T cell clone was added (2×10^4/well) together with 1 IU rIL-2/ml. Forty-eight hours later, 100 μl supernatant was removed and assayed for IFNγ as described above. The cells were then pulsed with ^{3}H-thymidine for 12–18 hr as described above to measure proliferation. The proliferation results are presented here.

Peptide Binding Assay

The peptide binding to affinity-purified H2A^b molecules was tested as described before (Harris et al., 1995). Briefly, H2A^b molecules (0.5 μM) purified from A20 cells transfected with H2A^b (Jurcevic et al., 1996) were mixed with 0.8 μM of biotinylated CLIP peptide (Ii 104–119, VSKMRMATPLLMQALP) and 0–1000 μM competitor peptide in a total volume of 30 μl of binding buffer (0.1 M sodium citrate, 30 mM octyl glucopyranoside, pH 6.0). After incubation at room temperature for 48 hr, the mixture was diluted in blocking buffer (PBS, 5% nonfat dry milk, 0.05% Tween-20), divided into triplicate sets, and transferred to a 96-well microtiter plate precoated with anti-H2A^b antibody (AF6–120.1.2, from American Tissue Culture Collection). After 2 hr incubation, the plates were washed, and the amount of H2A^b-bound biotinylated peptide was determined colorimetrically using streptavidine-peroxidase (Sigma, Poole, UK). Typically, absorbance in the presence of the biotinylated indicator peptide alone was in the range of 0.2–0.4, while the background was 0.01–0.02. The values are presented as either percent of inhibition of the binding of indicator peptide or 50% inhibitory peptide concentrations (IC50) calculated from regression analyses of absorbance values.

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