product was similar to *WT1-201*, a Gly → Asp mutation in *WT1* exon 3. To test the transactivational properties of *WT1-del2*, we used transient transfections of NIH 3T3 cells, in which *WT1* has been shown to act as a transcriptional repressor of the early growth response promoter (*EGR1*) (*18*). As expected, transfection of wild-type *WT1* repressed base line *EGR1*-CAT (chloramphenicol acetyltransferase) activity to one-fourth its original level. In contrast, transfection of *WT1-del2* led to twofold greater transcriptional activation (Fig. 2C). WT1 proteins with NH_2-terminal alterations [both synthetic deletion constructs as well as naturally occurring point mutants such as *WT1-201* and *WT1-273* (*9, 19*)] have also been shown to function as transcriptional activators; these results suggest that distinct domains of WT1 are responsible for transcriptional activation and repression. Cotransfection of equal amounts of wild-type *WT1* and *WT1-del2* resulted in an intermediate level of transactivation, which indicates a codominant effect by the two constructs. The antagonistic effect of wild-type *WT1* and *WT1-del2* suggests that *WT1* function may be disrupted in the fraction of Wilms tumors that express the largest amounts of this aberrant splicing product. Inactivation of gene function by expression of an alternatively spliced isoform has been described for a number of transcription factors (*20, 21*). For transcription factor E3, the alternatively spliced exon also resides within the transactivation domain (*21*), which suggests a functional antagonism analogous to that of *WT1-del2*. Thus, aberrant splicing of *WT1*, resulting in a protein with altered transactivational properties, represents a distinct and apparently common mechanism of *WT1* inactivation in Wilms tumors. Growth suppression by wild-type *WT1* of a Wilms tumor cell line expressing this altered endogenous transcript is consistent with the characterization of *WT1* as a tumor suppressor gene.

REFERENCES AND NOTES

1. H.-J. S. Huang *et al.*, *Science* **242**, 1563 (1988); R. Bookstein, J.-Y. Shew, P.-L. Chen, P. Scully, W.-H. Lee, *ibid.* **247**, 712 (1990); S. J. Baker, S. Markowitz, E. R. Fearon, J. K. V. Willson, B. Vogelstein, *ibid.* **249**, 912 (1990); L. Diller *et al.*, *Mol. Cell. Biol.* **10**, 5772 (1990); P.-L. Chen, Y. Chen, R. Bookstein, W.-H. Lee, *Science* **250**, 1576 (1990).
2. D. Haber and D. Housman, *Adv. Cancer Res.* **59**, 41 (1992).
3. S. Park *et al.*, *Nature Genet.*, in press.
4. D. Hazen-Martin, A. Garvin, T. Gansler, B. Tarnowski, D. Sens, *Am. J. Pathol.* **142**, 893 (1993); D. Hazen-Martin, G. Re, A. J. Garvin, D. A. Sens, unpublished data.
5. D. Haber *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9618 (1991).
6. D. Haber *et al.*, *Cell* **61**, 1257 (1990).
7. F. J. Rauscher III, J. F. Morris, O. E. Tournay, D. M. Cook, T. Curran, *Science* **250**, 1259 (1990); W. A. Bickmore *et al.*, *ibid.* **257**, 235 (1992).
8. D. Haber, H. Timmers, J. Pelletier, P. Sharp, D. Housman, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6010 (1992).
9. S. Park, G. Tomlinsen, P. Nisen, D. A. Haber, *Cancer Res.* **53**, 4757 (1993).
10. The two RM1 cell lines with the greatest expression of transfected *WT1*, NB2 (isoform B) and ND1 (isoform D), and cells mock-transfected with *neo* were seeded (10^4 cells each) in 0.34% low melting point agarose in culture medium over a cushion of 0.5% agarose in culture medium. After 6 weeks, the colonies were stained with the vital dye *p*-iodonitrotetrazolium violet (Sigma) and counted. The mean number of colonies per plate (±SD) was 1300 (±160) for *neo* cells, 320 (±80) for NB2 cells, and 280 (±80) for ND1 cells.
11. Six nude mice were subcutaneously injected with *neo*, NB2, or ND1 cells (10^8 each). At 21 days, subcutaneous tumors had developed in six of six *neo* mice, five of six NB2 mice, and four of six ND1 mice. The growth rate of the tumors differed between transfected cell lines. At 21 days, the mean maximum tumor diameter was 22 mm for *neo* mice, 16 mm for NB2 mice, and 10 mm for ND1 mice. All tumors were histologically identical and continued to express small amounts of the transfected construct.
12. B. E. Weissman *et al.*, *Science* **236**, 175 (1987); S. F. Dowdy *et al.*, *ibid.* **254**, 293 (1991); M. Koi *et al.*, *ibid.* **260**, 361 (1993).
13. A. Garvin, G. Re, B. Tarnowski, D. Hazen-Martin, D. Sens, *Am. J. Pathol.* **142**, 375 (1993).
14. K. Pritchard-Jones *et al.*, *Nature* **346**, 194 (1990); A. Buckler, J. Pelletier, D. Haber, T. Glaser, D. Housman, *Mol. Cell. Biol.* **11**, 1707 (1991).
15. A riboprobe spanning *WT1* exons 2 and 3 was annealed with total cellular RNA (10 μg) from different Wilms tumor cell lines and from fetal kidney. Ribonuclease protection analysis demonstrated a truncated transcript, consistent with the absence of exon 2, which accounted for all the detectable *WT1* mRNA in RM1 cells and ~50% of the *WT1* mRNA in RM14 cells. The truncated transcript was absent from normal fetal kidney.
16. G. Screaton *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12160 (1992).
17. J. Valcárcel, R. Singh, P. O. Zamore, M. R. Green, *Nature* **362**, 171 (1993).
18. S. L. Madden *et al.*, *Science* **253**, 1550 (1991).
19. Z.-Y. Wang, Q.-Q. Qiu, T. R. Deuel, *J. Biol. Chem.* **268**, 9172 (1993); S. Park *et al.*, *Nature Genet.* **4**, 415 (1993).
20. Y. Nakabeppu and D. Nathans, *Cell* **64**, 751 (1991); J. Yen and I. Verma, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5077 (1991); K. Yamamoto, G. Gonzalez, P. Menzel, J. Rivier, M. Montminy, *Cell* **60**, 611 (1990).
21. C. Roman, L. Cohn, K. Calame, *Science* **254**, 94 (1991).

22. We thank V. Sukhatme for providing the *EGR1-CAT* reporter construct and K. Isselbacher, E. Harlow, N. Dyson, J. Settleman, and S. Friend for critically reviewing our manuscript. D.A.H. is supported by grants from NIH (CA58596), the James S. McDonnell Foundation, the Edward Mallinckrodt, Jr., Foundation, and the James Culpepper Foundation. S.M. is supported in part by the Massachusetts General Hospital Fund for Medical Discovery (Janssen), C.E. by the Deutsche Forschungsgemeinschaft, and A.J.G. by NIH grant CA37887.

20 July 1993; accepted 8 November 1993

Peptide Translocation by Variants of the Transporter Associated with Antigen Processing

Marie-Thérèse Heemels, Ton N. M. Schumacher, Kurt Wonigeit, Hidde L. Ploegh*

Major histocompatibility complex (MHC) class I molecules associate with peptides that are delivered from the cytosol to the lumen of the endoplasmic reticulum by the transporter associated with antigen processing (TAP). Liver microsomes of SHR and Lewis rats, which express different alleles of TAP (*cim*^{b} and *cim*^{a}, respectively), accumulate different sets of peptides. Use of MHC congenic rats assigned this difference to the MHC, independent of the class I products expressed. Both the *cim*^{a} and *cim*^{b} TAP complexes translocate peptides with a hydrophobic carboxyl terminus, but translocation of peptides with a carboxyl-terminal His, Lys, or Arg residue is unique to *cim*^{a}. Thus, the specificity of the TAP peptide translocator restricts the peptides available for antigen presentation.

MHC class I molecules present peptides to antigen-specific receptors on $CD8^+$ T lymphocytes. These peptides are eight to ten residues in length and are acquired by newly synthesized MHC class I molecules in the lumen of the endoplasmic reticulum (ER) (*1*). The source of these peptides is mostly cytosolic (*1, 2*), and their delivery to the lumen of the ER is mediated by the TAP transporter (*3*). The TAP complex is a member of the adenosine triphosphate (ATP)–binding cassette family of transporters and consists of two subunits, TAP1 and TAP2 (*3*). Both subunits span the membrane six to eight times and have an ATP binding site in the proposed cytosolic domain (*3*). TAP-mediated translocation of peptides across the ER is temperature- and ATP-dependent (*4–6*). Little is known about the peptide substrate specificity of this transport process, although a role for the COOH-terminal residue of the peptide has been proposed (*5, 6*). Allelic variants of TAP1 and TAP2 might have different peptide substrate specificities, a speculation fueled by the location of both TAP genes

M.-Th. Heemels, T. N. M. Schumacher, H. L. Ploegh, Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.
K. Wonigeit, Medizinische Hochschule Hannover, Klinik fur Abdominal-und Transplantations Chirurgie, 30625 Hannover, Germany.

*To whom correspondence should be addressed.

within the polymorphic MHC. For the mouse and human TAP complex only a very limited degree of polymorphism has been observed thus far (*7*). In contrast, for the rat there is considerable sequence variation for different TAP alleles (*8*).

Polymorphism of the TAP genes alters the antigenic and biochemical properties of the rat class I molecule RT1A^a (the so-called class I modifier, or *cim* phenomenon) (*8*, *9*). Two distinct *cim* phenotypes exist: In the presence of the *cim*^{a} allele the RT1A^a class I molecule is efficiently transported to the cell surface, but the presence of the *cim*^{b} allele results in impaired transport and the inability of RT1A^a to present certain T cell epitopes (*8*, *9*). The distribution of this trait among different strains has been documented (*10*). The four TAP2 alleles identified by sequence analysis can unambiguously be assigned to the *cim*^{a} or *cim*^{b} type (*8*), as defined by immunological and biochemical experiments. The peptides associated with RT1A^a class I molecules synthesized by cells of *cim*^{a} and *cim*^{b} type show different elution profiles on high-pressure liquid chromatography (HPLC). The peptides provided by the *cim*^{a} background appear more hydrophilic (*8*). Here we determine the substrate specificity of the *cim*^{a} and *cim*^{b} alleles of the rat transporter and show how polymorphism in the peptide transporter can affect the spectrum of peptides available to class I molecules.

Liver microsomes can accumulate peptides in a TAP- and ATP-dependent manner. For translocation to be measured, a retention device for translocated peptides must be present on the lumenal side of the ER (*6*). This can either be a class I molecule capable of interacting with the peptide (*6*) or the attachment of an N-linked oligosaccharide to the peptide on the lumenal side of the ER. When microsomes prepared from rat liver were incubated with the iodinated substrate TYQRTRALI (*11*), only small amounts of this peptide accumulated inside (Fig. 1A, left panel). Substitution of the Gln residue with Asn creates a peptide, TYNRTRALI, that can be N-glycosylated. This substrate is translocated and retained by microsomes in a temperature-, ATP- and time-dependent fashion (Fig. 1A, right panel). After 10 to 15 min at 37°C, accumulation reaches a plateau. Analysis by gel electrophoresis (*12*) of peptides retained by the microsomes shows that all peptides contain an endoglycosidase H–sensitive glycan (Fig. 1B) (*11*). The kinetics of this ATP- and temperature-dependent accumulation of peptides are strictly comparable to those reported for TAP-dependent peptide transport into mouse microsomes (*4*). Immunoprecipitation from detergent extracts of peptide-loaded microsomes with the monomorphic monoclonal antibody to rat MHC class I molecules, OX18 (*14*), did not result in recovery of radioactivity over background. Class I molecules could be readily immunoprecipitated under similar conditions from biosynthetically labeled spleen cells. Therefore, accumulation of peptide is not mediated by an interaction with MHC class I molecules.

The ability of COOH-terminal variants of the TYQRTRALI peptide (TYQRTRALX, where X is any amino acid except Cys or Pro) to compete for accumulation of radiolabeled TYNRTRALI peptide was assessed (*15*). Microsomes were prepared from SHR (*cim*^{b}) and Lewis (*cim*^{a}) rats (Table 1) (*10*). For the *cim*^{b} microsomes (Fig. 2A), the peptides that compete best for uptake of the reporter substrate are those terminating in Tyr, Trp, or Phe, followed by those ending in Met, Leu, Ile, and Val. Comparable results were obtained with microsomes prepared from Brown-Norway rats, which also carry the *cim*^{b} type of transporter but express different class I alleles (Table 1). A similar preference for hydrophobic COOH-termini has been observed for the mouse transporter (*6*). In contrast, microsomes of *cim*^{a} origin (Fig. 2A) can translocate peptides ending in aromatic and aliphatic residues and peptides with a basic COOH-terminal residue (His, Lys, or Arg) and to a lesser extent other polar residues such as Gln and Thr. A comparison of the different competitor peptides at 10 μM for both *cim* alleles clearly illustrates the difference in specificity (Fig. 2B). The use of congenic rat strains, listed in Table 1, allowed unequivocal assignment of this difference in translocation capacity to the MHC locus, in strict correlation with their known *cim* type and independent of the class I alleles expressed (Fig. 2C). On the whole, the *cim*^{a} transporter appears to be less selective than the *cim*^{b} transporter in terms of the COOH-termini it will tolerate in competitor peptides. Peptides ranging

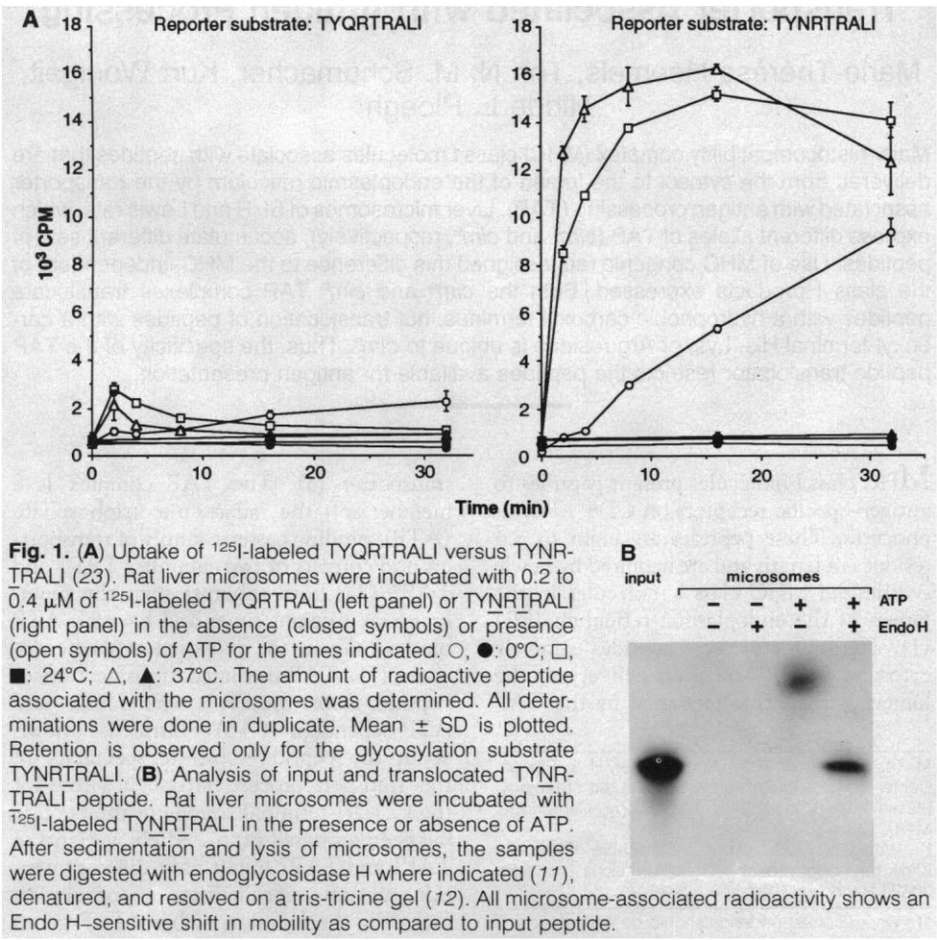

Fig. 1. (**A**) Uptake of ^{125}I-labeled TYQRTRALI versus TYNRTRALI (*23*). Rat liver microsomes were incubated with 0.2 to 0.4 μM of ^{125}I-labeled TYQRTRALI (left panel) or TYNRTRALI (right panel) in the absence (closed symbols) or presence (open symbols) of ATP for the times indicated. ○, ●: 0°C; □, ■: 24°C; △, ▲: 37°C. The amount of radioactive peptide associated with the microsomes was determined. All determinations were done in duplicate. Mean ± SD is plotted. Retention is observed only for the glycosylation substrate TYNRTRALI. (**B**) Analysis of input and translocated TYNRTRALI peptide. Rat liver microsomes were incubated with ^{125}I-labeled TYNRTRALI in the presence or absence of ATP. After sedimentation and lysis of microsomes, the samples were digested with endoglycosidase H where indicated (*11*), denatured, and resolved on a tris-tricine gel (*12*). All microsome-associated radioactivity shows an Endo H–sensitive shift in mobility as compared to input peptide.

Table 1. MHC haplotype and *cim* allotype for the strains of rat used.

Rat strain	MHC haplotype	*cim* allele
Lewis	RT1^l	*cim*^{a}
Lewis.1N	RT1^n	*cim*^{b}
Lewis.1W	RT1^u	*cim*^{b}
Brown Norway	RT1^n	*cim*^{b}
Brown Norway.1L	RT1^l	*cim*^{a}
SHR	RT1^k	*cim*^{b}

from 6 to 13 residues in length (RTRALV, QRTRALV, YQRTRALV, TYQRTRALV, GTYQRTRALV, GTTYQRTRALV, GGTTYQRTRALV, and GGTTTYQRTRALV) were examined for the ability to compete for uptake of TYNRTRALI. Only peptides longer than seven to eight residues are capable of inhibition, with a preference for peptides containing nine and ten residues. The length preference of *cim*^{a} and *cim*^{b} allelic variants of the transporter is similar for the set of peptides tested.

Although the COOH-terminal residue of a peptide can be crucial to substrate recognition by the TAP translocator, the contribution of sequence elements of the peptide other than the COOH-terminus remained to be determined. Two peptide libraries of limited complexity, but encompassing a wide range of charges and hydrophobicity, were synthesized (*16*). A library of 384 peptides (charge range, −3 to +5), all terminating in Ile, and a library containing 2304 peptides (charge range, −4 to +6), terminating in Ile, His, Lys, Asp, Ala, or Phe (Fig. 3A), were used.

Microsomes of *cim*^{a} and *cim*^{b} type were incubated with the COOH-terminal variable library (n = 2304), and peptides retained by the vesicles were separated by reverse-phase HPLC. A marked difference in elution profile is seen for peptides retained by *cim*^{a} and *cim*^{b} microsomes (Fig. 3C) (*17*). As predicted by their ability to translocate peptides terminating in His, Lys, and Arg, the *cim*^{a} microsomes select a more hydrophilic (earlier eluting) population than do *cim*^{b} microsomes. This result is reminiscent of the data obtained for RT1A^a class I–eluted peptides synthesized in a *cim*^{a} or *cim*^{b} background (*8*). Similarly, analysis by nonequilibrium pH gradient electrophoreses (NEPHGE) (Fig. 3B, right panel) (*17*) shows that the most basic peptides present in the starting population are translocated by *cim*^{a}, but not *cim*^{b}, microsomes. Thus, *cim*^{b} selects, from a set of peptides that contain considerable variation in charge and hydrophobicity, a subset of the peptides that are translocated by the *cim*^{a} transporter.

The contribution to substrate specificity of residues other than the COOH-terminal residue was examined. The *cim*^{a} and *cim*^{b} microsomes were incubated with the peptide library containing a fixed COOH-terminal amino acid that is permissive for both TAP alleles (Ile, n = 384). By NEPHGE no obvious differences were observed for *cim*^{a} and *cim*^{b} microsomes in the pattern of retained peptides (Fig. 3B, left panel). However, analysis of microsome-associated peptides by HPLC revealed a slight displacement of the profile toward more hydrophilic peptides for those retained by the *cim*^{a} microsomes (Fig. 3D). This observation is consistent with a contribution of features other than the COOH-terminus of the peptide in determining translocation. Not all peptides in the input material migrate at the same position in NEPHGE as the peptides retained by the microsomes (Fig. 3B). This difference may represent selection, by the transporter, of a subset of peptides, a suggestion not immediately confirmed by HPLC (Fig. 3, C and D). Alternatively, the presence of the Asn-linked glycan may affect the pK_a of charged residues in its vicinity, may affect the mobility of peptides in NEPHGE for hydrodynamic reasons, or both.

Is the ability to translocate peptides that terminate in His, Lys, or Arg and peptides with a hydrophobic COOH-terminus determined by a single site on the peptide translocator? In *cim*^{b} microsomes, only TYQRTRALI is capable of inhibiting uptake of both libraries. In contrast, both TYQRTRALI and TYQRTRALR prevent uptake of the libraries by *cim*^{a} microsomes (Fig. 4). These results are consistent with the notion that a single substrate acceptor site in the *cim*^{a} transporter accommodates peptides ter-

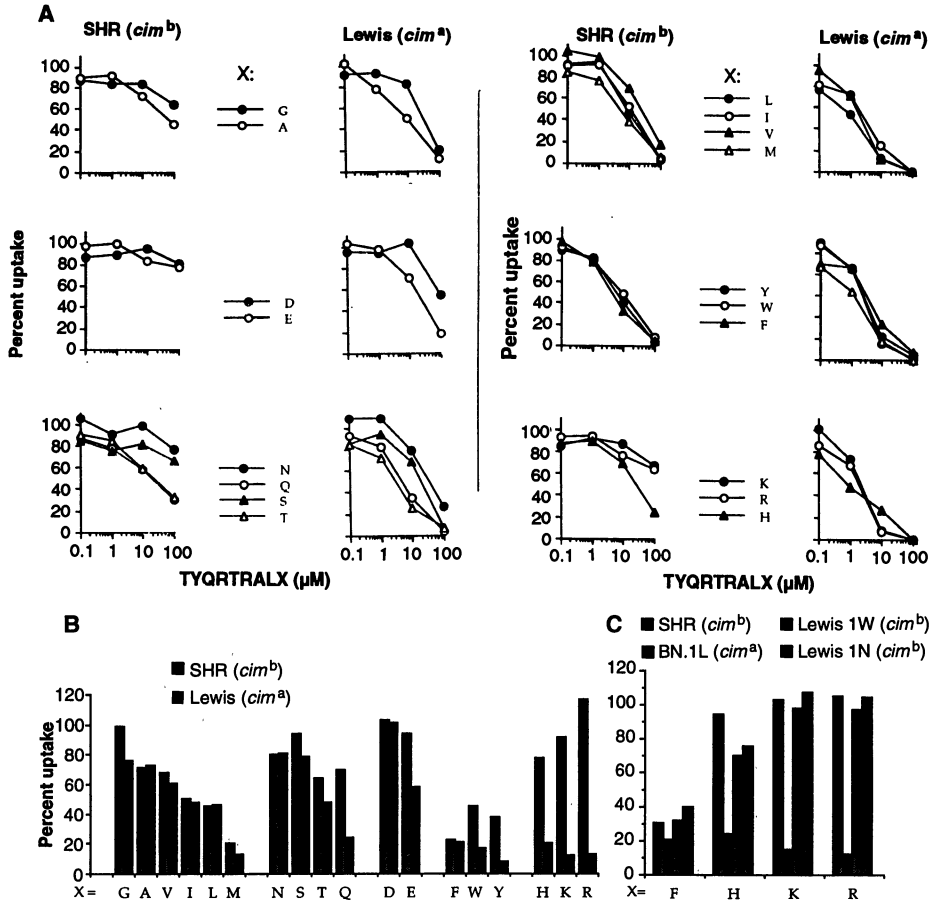

Fig. 2. (**A**) The *cim*^{a} and *cim*^{b} microsomes prefer peptides with a different COOH-terminus. Microsomes derived from SHR (*cim*^{b}; left columns) and Lewis (*cim*^{a}; right columns) rats were used for competition assays with ^{125}I-TYNRTRALI reporter substrate and unlabeled TYQRTRALX peptides as competitors (at the concentrations indicated). The identity of X is indicated between the columns in single-letter code. For each experiment, uptake in absence of competitor peptide was set at 100% (*11*), and translocation in the presence of competitor was expressed relative to this value. Two different batches of radiolabeled peptide were used for the competition studies with SHR and Lewis microsomes, respectively, allowing a qualitative comparison of SHR and Lewis microsomes only. (**B**) Comparison of *cim*^{a} and *cim*^{b} transporter specificity at 10 μM competitor peptide, with a single batch of reporter substrate. Data were obtained at 10 μM competitor TYQRTRALX in the type of experiment shown in Fig. 2A. Note the difference between *cim*^{a} and *cim*^{b} transporter specificity in particular for peptides terminating in His, Lys, and Arg, and to a lesser extent in Gln and Glu. (**C**) Assignment of translocation specificity to the MHC and in accordance with *cim* type. Peptide uptake was compared for SHR (*cim*^{b}), Brown-Norway 1L (*cim*^{a}), and RT1 congenics on the Lewis background: Lewis 1W (*cim*^{b}) and Lewis 1N (*cim*^{b}). Transfer of the *cim*^{b} allele to the Lewis background abrogates the ability to translocate peptides with a COOH-terminal His, Lys, or Arg methods as in Fig. 2B. Transfer of the *cim*^{a} allele to the Brown-Norway background results in translocation of these same peptides.

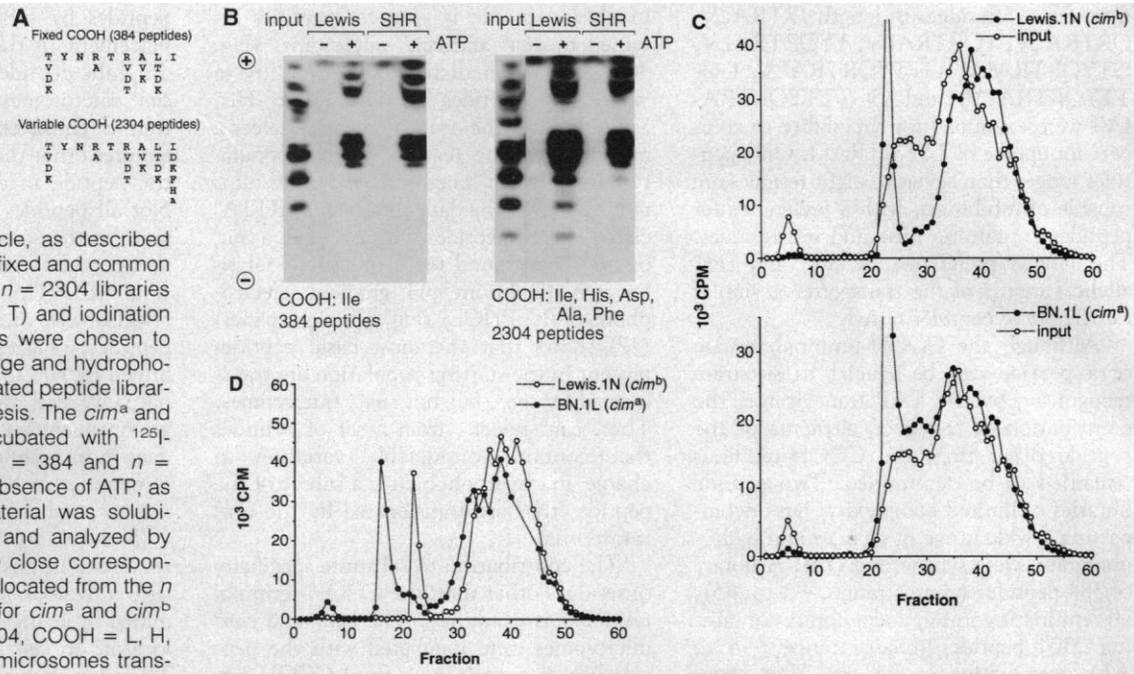

Fig. 3. (**A**) Synthesis of peptide libraries. Scheme used for synthesis of library with a fixed COOH-terminal Ile (top) and a library with a variable COOH-terminus (bottom). The vertically aligned amino acids were incorporated in a single coupling cycle, as described (*16*). Three amino acids are fixed and common to both the n = 384 and the n = 2304 libraries to allow for glycosylation (N, T) and iodination (Y) (*11*). The other residues were chosen to maximize differences in charge and hydrophobicity. (**B**) Input and translocated peptide libraries analyzed by electrophoresis. The *cim*^{a} and *cim*^{b} microsomes were incubated with ^{125}I-labeled peptide libraries (n = 384 and n = 2304) in the presence and absence of ATP, as indicated. Translocated material was solubilized in IEF sample buffer and analyzed by NEPHGE gel (*11*). Note the close correspondence for the peptides translocated from the n = 384, COOH = Ile library for *cim*^{a} and *cim*^{b} microsomes. For the n = 2304, COOH = L, H, D, A, F, K library, the *cim*^{a} microsomes translocate a set of peptides that are absent from the material translocated by *cim*^{b} microsomes. These are peptides with high electrophoretic mobility toward the cathode and therefore represent the most basic peptides. (**C**) Peptides selected from a library with a variable COOH-terminal residue analyzed by HPLC. The ^{125}I-labeled (n = 2304) peptide library was used in a translocation experiment with *cim*^{b} (upper panel) and *cim*^{a} (lower panel) microsomes. Microsome-associated peptides were extracted with TFA and resolved by HPLC. Fractions were collected, and radioactivity was determined. Shown are elution profiles for microsome-associated peptides (closed symbols) and input peptide library (open symbols). (Upper panel) Note the lack of coincidence in input and translocated material between fractions 20 through 35, and the slight displacement (as compared to input) toward late-eluting (hydrophobic) peptides for the translocated fraction. (Lower panel) Note the close fit between input peptides (open symbols) and translocated peptides (closed symbols), and especially the preponderance of early-eluting (hydrophilic) peptides (*17*). The profiles of the internal cold peptide library standards were identical. (**D**) Peptides selected from a library with a fixed COOH-terminal residue analyzed by HPLC. The ^{125}I-labeled COOH-terminal Ile (n = 384) library was used in a translocation assay. The elution profile is shown for peptides extracted from Lewis.1N (*cim*^{b}; closed symbols) and BN.1L (*cim*^{a}; open symbols) microsomes. Note the slight displacement of the profile for *cim*^{a} microsomes toward earlier eluting peptides. This displacement was observed in three independent comparisons of *cim*^{a} and *cim*^{b} microsomes. The identities of the early-eluting peaks (fractions 15 through 18 and 22 and 23, respectively) have not been established.

minating in Arg or Ile, but the existence of multiple recognition sites that cannot be utilized simultaneously remains a possibility.

It is concluded that in the rat, allelic variation in TAP greatly affects the specificity of the peptide translocation process. Sequence elements other than the COOH-terminal peptide residue can contribute to the ability of a peptide to be translocated by *cim*^{a} or *cim*^{b} transporters. Such elements remain to be defined in structural terms, while the overriding importance of the COOH-terminal position is clear. In the mouse, all residues observed at the COOH-terminus of peptides bound by class I molecules are hydrophobic (*2*). The mouse TAP complex also prefers a hydrophobic COOH-terminus in its substrates (*6*), a property it shares with rat transporters of *cim*^{b} type. In human, class I–bound peptides have either a hydrophobic or a positively charged COOH-terminus (*2*, *18*). If a similar link exists between the COOH-termini of class I–bound peptides and the specificity of the human TAP complex, the human transporter must resemble the rat *cim*^{a} transporters. Because the presence of a *cim*^{a} transporter is necessary for efficient surface expression of, and antigen presentation by, RT1A^a (*8*, *9*), we suggest that the RT1A^a molecule may prefer peptides with a positively charged COOH-terminus.

For recombinant strains, where the RT1A^a class I molecule is served by the TAP *cim*^{b} allele, the transporter clearly is a restriction point in antigen presentation. However, strains of rat that are RT1A^a invariably carry the *cim*^{a} allele, excepting the laboratory-bred rats in which the RT1A locus was dissociated from its usual transporter by recombination (*8*–*10*). Thus, although the TAP complex limits the spectrum of peptides available in the ER (*19*), natural selection may have resulted in a situation that avoids restriction by the

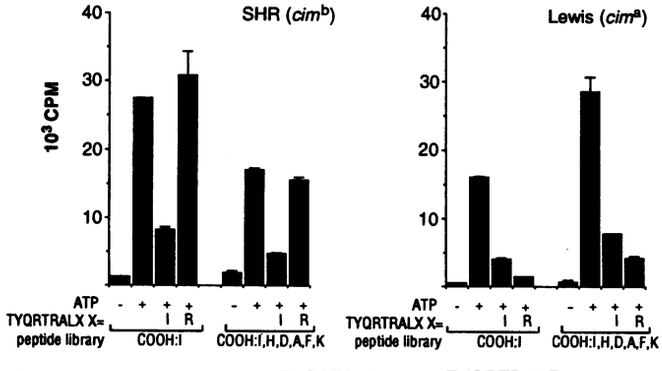

Fig. 4. Peptides with different COOH-termini compete for the same site on TAP. Competition experiments were performed with 10 μM TYQRTRALI or TYQRTRALR, as indicated, with the ^{125}I-labeled peptide libraries (COOH:I, n = 386) and COOH:I,H, D,A,F,K; n = 2304) as reporter substrates. In *cim*^{b} microsomes (left panel), only TYQRTRALI is an effective competitor, whereas in *cim*^{a} microsomes, both TYQRTRALI and TYQRTRALR are good competitors. In *cim*^{a} microsomes, therefore, peptides with different COOH-termini are most likely accommodated by a single site.

transporter by matching the specificity of the class I molecules and the transporters that serve them.

REFERENCES AND NOTES

1. A. Townsend and H. Bodmer, *Ann. Rev. Immunol.* **7**, 601 (1989); M.-J. Bijlmakers and H. L. Ploegh, *Curr. Opin. Immunol.* **5**, 21 (1993); A. L. Goldberg and K. L. Rock, *Nature* **357**, 375 (1992).
2. H.-G. Rammensee, K. Falk, O. Rotzschke, *Ann. Rev. Immunol.* **11**, 213 (1993).
3. P. Parham, *Nature* **348**, 674 (1990); T. Spies *et al.*, *ibid.*, p. 744; J. Trowsdale *et al.*, *ibid.*, p. 741; E. V. Deverson *et al.*, *ibid.*, p. 738; J. J. Monaco *et al.*, *Science* **250**, 1723 (1990).
4. J. C. Shepherd *et al.*, *Cell* **74**, 577 (1993).
5. J. J. Neefjes, F. Momburg, G. Hammerling, *Science* **261**, 769 (1993).
6. T. N. M. Schumacher *et al.*, *J. Exp. Med.*, in press.
7. H. R. Gaskins *et al.*, *Science* **256**, 1826 (1992); M. Colonna *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3932 (1992).
8. S. J. Powis *et al.*, *Nature* **357**, 211 (1992).
9. A. M. Livingstone *et al.*, *J. Exp. Med.* **170**, 777 (1989); S. Powis *et al.*, *ibid.* **173**, 913 (1991).
10. A. M. Livingstone *et al.*, *Immunogenetics* **34**, 157 (1991).
11. After testing peptides of different sequence and lengths, we identified TYQRTRALI as a candidate for translocation assays in rat microsomes. The substitution Q to N was considered conservative. Microsomes were prepared from rat liver as described (*4, 20*), but were finally resuspended in buffer I (50 mM tetraethylammonium, 1.5 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Synthetic peptides (*6*) and peptide libraries (*16*) were synthesized and dissolved as described. Peptides were labeled by chloramine T–catalyzed iodination to a specific activity of 10 Ci/mmol. Competition assays were done as described (*4, 6*) with the following modifications: Incubation was done for 15 min at 37°C, and 0.1 to 0.4 μM of peptide (depending on the batch peptide) was used; iodinated TYNRTRALI was used as reporter substrate unless indicated otherwise. Assays were stopped by addition of 1 ml of ice-cold buffer NaI + 10 mM EDTA. Microsomes were sedimented (14,000*g* for 20 min at 4°C), and microsome-associated radioactivity was determined. Endoglycosidase H digestion and electrophoretic analysis were done as described (*13*). Percent uptake was calculated as follows:

$$\frac{[\text{CPM}(+\text{ATP};+\text{competitor})]-[\text{CPM}(-\text{ATP})]}{[\text{CPM}(+\text{ATP})]-[\text{CPM}(-\text{ATP})]}$$

Values are means of duplicates for which the standard deviation is less than 15% of the mean.
12. H. Schägger and G. Von Jägow, *Anal. Biochem.* **166**, 368 (1987).
13. M.-J. Bijlmakers *et al.*, *Eur. J. Immunol.* **23**, 1305 (1993).
14. T. Fukumoto, W. R. McMaster, A. F. Williams, *ibid.* **12**, 237 (1982).
15. TYQRTRALX competitor peptides were used instead of TYNRTRTALX, to avoid competition for glycosylation.
16. T. N. M. Schumacher *et al.*, *Eur. J. Immunol.* **22**, 1405 (1992).
17. Analysis by NEPHGE was done as described (*21*) with the following modifications: Ampholytes were pH 3.5 to 10 (2%) and pH 5 to 7 (0.4%); samples were loaded at the anodal side of the gel and run for 2.5 hours at 400 V. The gel was frozen and exposed to Kodak X-AR5 film. Trifluoroacetic acid (TFA) extracts of microsomal pellets (2 × 1% TFA) were analyzed by HPLC as described (*22*) with a reversed-phase C18 column [3 min constant at 5%, increase in 50 min from 5% to >55% (v/v) acetonitrile in 0.1% TFA]. Fractions of 0.5 min were collected, and radioactivity was determined. The identity of the peak in the translocated material (fractions 22 to 24) has not been established. The height of this peak was found to vary between experiments, and the material is not obviously present in input peptides.
18. T. S. Jardetzky *et al.*, *Nature* **353**, 326 (1991); H.-C. Guo *et al.*, *ibid.* **360**, 364 (1992).
19. J. C. Howard, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3777 (1993).
20. P. Walter and G. Blobel, *Methods Enzymol.* **96**, 84 (1983).
21. J. J. Neefjes *et al.*, *Hum. Immunol.* **16**, 169 (1986).
22. T. J. Tsomides and H. N. Eisen, *Anal. Biochem.* **210**, 129 (1993).
23. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

24. We acknowledge the support of R. Cook and M. Kelly of the Massachusetts Institute of Technology Biopolymers facility for synthesis of peptide libraries. We thank members of the DCM, M. Ullman-Culleré, and S. Robinson of the Hynes lab for assistance in handling the animals; A. Bandeira for revealing the identity of the rats; T. Tsomides for help in HPLC separations; and D. Kantesaria and other members of the Ploegh lab for reading the manuscript. Supported by NIH grant RO1 AI3 3456-01 and the Dutch Foundation for Rheumatism.

15 October 1993; accepted 30 November 1993

TECHNICAL COMMENTS

Male Sexual Orientation and Genetic Evidence

Dean H. Hamer *et al.* report a paired sibling (sib-pair) analysis linking male sexual orientation with a region of chromosome Xq28 (*1*). Much of the discussion of this finding has focused on its social and political ramifications. In contrast, our goal is to discuss the scientific evidence and to highlight inconsistencies that suggest that this finding should be interpreted cautiously.

While interpretation of genetic linkage results for Mendelian traits (such as genetic markers) by lod score statistics has a substantial history, including empirical verification, this is not so for complex, or non-Mendelian traits. There are pitfalls in applying standards derived for Mendelian traits to complex ones (*2*). Interpretation of linkage results for such a trait must, of necessity, occur in the context of prior evidence about the genetic basis for that trait. Family, twin, and adoption studies, the basic paradigms of genetic epidemiology, provide important evidence about the magnitude and nature of a possible genetic contribution to a trait. Furthermore, quantifying the magnitude of a genetic effect, through family and adoption studies, plays a direct role in the interpretation of linkage results. This is because there is a simple correspondence between the familiality of a trait that results from a gene and the expected linkage evidence (*3*). Specifically, inheritance of alleles identical by descent (ibd) at an autosomal contributory locus (or closely linked marker) for an affected (or trait-concordant) sib pair is directly related to the ratio (λ_s) of recurrence for siblings over population prevalence (*3*)

$$z_0 = 0.25/\lambda_s$$

where z_0 is the probability that the sib-pair share no alleles ibd at the trait locus, and λ_s is the sibling recurrence ratio that results from that locus. For an X-linked locus and brother pairs, the equivalent formula is

$$z_0 = 0.50/\lambda_s$$

Hence, interpretation of ibd data for trait-concordant brother pairs depends on prior evidence about plausible values of λ_s for such a locus.

Hamer *et al.* (*1*) and two other recent studies (*4, 5*) suggest the existence of an increased frequency of homosexual orientation among the brothers of gay probands; however, the one study (*5*) that included monozygous (MZ) and dizygous (DZ) cotwins, as well as adopted (unrelated) and biological brothers of homosexual probands, provided inconsistent genetic evidence. While the MZ cotwins showed a higher rate than DZ cotwins of homosexual orientation (29/56 or 52%, as opposed to 12/54 or 22%, respectively), which suggested a genetic contribution, the biological brothers and adoptive brothers showed approximately the same rates (13/142 or 9.2% and 6/57 or 10.5%, respectively). This latter observation suggests that there is no genetic component, but rather an environmental component shared in families. Hence, one's interpretation of the results of this study (*5*) depends on whether one places greater credibility on the data from twins or on the data from adopted and biological siblings. The fact that the study population was obtained through advertisement may have also led to undetermined biases.

From these three studies, it is somewhat difficult to obtain an accurate (or consistent) estimate of λ_s. To calculate λ_s an appropriate control group is required. For controls, Pillard and Weinrich (*4*) studied the brothers of heterosexual probands and obtained a rate of homosexual orientation of 2/38 or 5.3% for those directly interviewed, compared with a rate of 15/68 or 22% for the brothers of gay probands. From these data, we obtain a value of λ_s =