21. E. Vegeto *et al.*, *Cell* **69**, 703 (1992); J. M. Beekman, G. F. Allan, S. Y. Tsai, M.-J. Tsai, B. W. O'Malley, *Mol. Endocrinol.* **7**, 1266 (1993).
22. C. L. Smith, O. M. Conneely, B. W. O'Malley, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6120 (1993).
23. V. Giguere, S. M. Hollenberg, M. G. Rosenfeld, R. M. Evans, *Cell* **46**, 645 (1986); K. Umesono, K. K. Murakami, C. C. Thompson, R. M. Evans, *ibid.* **65**, 1255 (1991); X. Leng *et al.*, *J. Biol. Chem.* **269**, 31436 (1994); A. J. Cooney, X. H. Leng, S. Y. Tsai, B. W. O'Malley, M.-J. Tsai, *ibid.* **268**, 4152 (1993); K. Helin, E. Harlow, A. Fattaey, *Mol. Cell. Biol.* **13**, 6501 (1993); A. J. Courey and R. Tjian, *Cell* **55**, 887 (1988).
24. A. Baniahmad, A. C. Kohne, R. Renkawitz, *EMBO J.* **11**, 1015 (1992).
25. F. M. Ausubel *et al.*, *Short Protocols in Molecular Biology* (Greene and Wiley, New York, 1992).
26. L. A. Denner, N. L. Weigel, B. L. Maxwell, W. T. Schrader, B. W. O'Malley, *Science* **250**, 1740 (1990).

27. We would like to thank S. Law for his assistance in sequencing SRC-1 and P. Samora for his technical assistance. We also thank S. Elledge for providing the materials for the yeast two-hybrid system and M. Montminy for providing CREB expression vector and CREB response DNA element. This work was supported by NIH grant HD08188 and American Cancer Society grant BE63.

9 July 1995; accepted 26 October 1995

Self-Release of CLIP in Peptide Loading of HLA-DR Molecules

Harald Kropshofer,* Anne B. Vogt,* Lawrence J. Stern, Günter J. Hämmerling†

The assembly and transport of major histocompatibility complex (MHC) class II molecules require interaction with the invariant chain. A fragment of the invariant chain, CLIP, occupies the peptide-binding groove of the class II molecule. At endosomal pH, the binding of CLIP to human MHC class II HLA-DR molecules was counteracted by its amino-terminal segment (residues 81 to 89), which facilitated rapid release. The CLIP(81–89) fragment also catalyzed the release of CLIP(90–105) and a subset of other self-peptides, probably by transient interaction with an effector site outside the groove. Thus, CLIP may facilitate peptide loading through an allosteric release mechanism.

The peptide-binding groove of MHC class II molecules is blocked in the endoplasmic reticulum by association with the invariant chain (Ii) (*1*). Proteolytic digestion of Ii in endosomal compartments generates CLIP (*2*), which encompasses the region of Ii involved in binding to MHC class II (*3*) and must be released from class II molecules before loading with peptide (*4*). The nonclassical MHC class II molecule HLA-DM has been implicated in the removal of CLIP from class II molecules (*5*, *6*). However, class II alleles differ with regard to their dependency on HLA-DM. Thus, for example, efficient removal of CLIP from DR3 and DR11 in vivo and subsequent peptide loading depend on the expression of HLA-DM (*4*), whereas no comparable requirement for HLA-DM is apparent for DR4Dw4, A^d, or A^k (*4*, *7*). In addition, loading with conventional self-peptides other than CLIP has been demonstrated in most DR^+DM^- mutant cells, with the proportion of non-CLIP peptides varying between different class II alleles (*2*–*4*). These observations suggest that CLIP removal does not depend absolutely on HLA-DM, even for alleles, such as A^d, that bind CLIP with high affinity (*8*, *9*).

The peptide repertoire of DR3 molecules from the DM-negative mutant cell line T2.DR3 was assessed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The repertoire was characterized by a clustering of signals with mass-to-charge ratios (m/z) of 1500 to 2300, indicative of conventional self-peptides, that accounted for ~30 to 50% of the total peptides in various DR3 preparations (Fig. 1). The remaining 50 to 70% of the peptides were accounted for by four prominent CLIP variants. In general, a smaller proportion of CLIP peptides (10 to 20%) is associated with DR3 from DM-positive, wild-type cells.

A possible explanation for the partial peptide loading of DR3 in DM-negative T2.DR3 mutants is that CLIP may facilitate its own dissociation from the class II molecule in the absence of HLA-DM. To test this hypothesis, we determined the kinetics of CLIP release from DR3 by performing in vitro peptide binding assays based on high-performance size-exclusion chromatography (HPSEC) (*10*). An important methodological parameter that influences CLIP dissociation is the detergent used (*4*). In a detergent-free system, labeled CLIP(81–105) dissociated rapidly from recombinant soluble DR3 and DR1 (sDR3 and sDR1) produced in insect cells, displaying a time of half-maximal dissociation, $t_{1/2}$, of ~2 hours at 37°C and pH 5.8 (Fig. 2A). NP-40, a detergent often used in binding assays involving CLIP, prevented rapid dissociation of CLIP, thereby explaining the slow rate of dissociation observed in other studies (*6*, *9*, *11*). In contrast, the detergent Zwittergent-12 (ZW-12) slightly increased the rate of CLIP dissociation. The high rate of CLIP dissociation was not a peculiarity of recombinant DR molecules; similar rates ($t_{1/2}$ of ~30 min in the presence of ZW-12) were observed with sDR3 and with DR3 from T2.DR3 cells (Fig. 2B). The rate of CLIP dissociation increased further at pH 4.8 ($t_{1/2} \approx 15$ min), whereas the antigenic peptide HSP65(3–13) remained stably bound at this pH ($t_{1/2} > 48$ hours) (Fig. 2B). Dissociation of endogenously bound CLIP variants from DR3 molecules isolated from T2.DR3 cells and depleted of detergent showed a time course similar to that for the sDR3:CLIP complexes generated in vitro ($t_{1/2} \approx 1.5$ hours), as revealed by MALDI-MS kinetics. Thus, DR3:CLIP complexes formed in vivo and in vitro display comparably low stabilities in the absence of detergent (*12*).

The COOH-terminal region of CLIP is thought to occupy the peptide-binding groove of MHC class II molecules in a manner similar to antigenic peptides (*9*, *11*), with the NH_2-terminal residues protruding from the groove. Both NH_2- and COOH-terminal extensions of antigenic peptides usually confer increased stability to MHC class II αβ:peptide complexes during SDS–polyacrylamide gel electrophoresis (*13*). However, long CLIP peptides containing NH_2-terminal extensions are released relatively rapidly from DR molecules

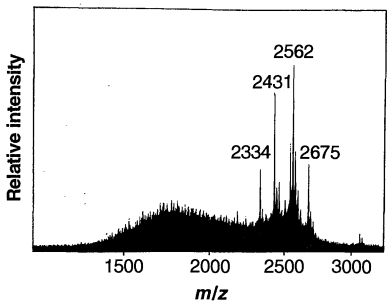

Fig. 1. Mass profile of DR3-associated self-peptides from T2.DR3 cells: Endogenous peptides released by acid treatment of 5 μg of affinity-purified DR3 molecules (*24*) from the human DM-negative mutant Epstein-Barr virus–transformed B/T hybrid cell line T2.DR3 (T2 cells transfected with DR3) were analyzed by MALDI-MS (*25*). The indicated masses correspond to CLIP(82–102) (m/z = 2334), CLIP(82–103) (m/z = 2431), CLIP(82–104) (m/z = 2562), and CLIP(81–104) (m/z = 2675) (*3*).

H. Kropshofer, A. B. Vogt, G. J. Hämmerling, Department of Molecular Immunology, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.
L. J. Stern, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.
*These authors contributed equally to this work.
†To whom correspondence should be addressed.

at low pH (*14*). We compared the dissociation rates of CLIP(81–105) and the shorter CLIP(90–105). Removal of the NH_2-terminal residues 81 to 89 stabilized the corresponding sDR3 complex, increasing $t_{1/2}$ from 1.5 to ~6 hours (Fig. 3A). Thus, residues 81 to 89 of CLIP appear to have a destabilizing effect. To determine which residues are responsible for this effect, we prepared single alanine substitutions of CLIP(81–105) (Fig. 3A). Complexes with peptides in which Lys^{83} or Pro^{87} was replaced with Ala showed an increased stability similar to that observed with CLIP(90–105); alanine substitution of Lys^{86} had a similar, but less pronounced, effect. These residues are conserved in the Ii sequences of rat, mouse, and human, whereas Pro^{84} and Pro^{85}, which have no effect on $t_{1/2}$, are replaced by Ser^{84} and Ala^{85} in rat and mouse (*15*). The data suggest that Lys^{83}, Lys^{86}, and Pro^{87} interact with an effector site on DR3 molecules and thereby induce a conformational rearrangement that destabilizes essential contacts between DR3 and the COOH-terminal region of CLIP.

We next investigated whether CLIP residues 81 to 89 are active only in the context of CLIP(81–105) or whether they can also destabilize complexes in a trans configuration. The addition of CLIP(81–89) to a preformed sDR3:CLIP(90–105) complex reduced the $t_{1/2}$ for dissociation of CLIP(90–105) from ~6 to 2 hours, approximately the same value as for dissociation of CLIP(81–105) (Fig. 3B). As a control, HSP65(3–13) had no effect on dissociation of CLIP(90–105). Substitution of Ala^{94} of CLIP by Asp facilitates interaction with an additional pocket in the peptide-binding groove and increases complex stability (*11*). Accordingly, CLIP(90–105)A94→D bound stably to sDR3 and showed a $t_{1/2}$ for dissociation of ~64 hours. The presence of CLIP(81–89) decreased the $t_{1/2}$ for CLIP(90–105)-A94→D to ~3.5 hours, which is similar to the value obtained with the corresponding long version of modified CLIP, CLIP(81–105)A94→D. In contrast, CLIP(81–89) had no effect on the dissociation rate of CLIP(81–105) or the stably bound antigenic peptide HSP65(3–13). However, CLIP(81–89) induced the dissociation of self-peptides from DR3 isolated from B-LCL COX cells, as revealed by MALDI-MS (Fig. 3C); the presence of CLIP(81–89) resulted in selective loss of a subset of peptides in the lower mass range, predominantly with m/z ratios of 1900 to 2000. We cannot exclude the possibility that the released peptide subset may contain some CLIP variants. However, the observation that CLIP(81–89) also reduced the $t_{1/2}$ for dissociation of the stably bound antigenic peptide MOMP(248–260) (*16*) from ~75 to 30 hours (Fig. 3B) indicates that CLIP(81–89) is able to increase the rate of dissociation of peptides other than CLIP. This conclusion is consistent with the recent observation that CLIP(77–92) enhances antigen presentation by promoting the exchange of peptides on the cell surface (*17*). In contrast, CLIP(81–89) had no effect on the dissociation of MOMP(246–260), a peptide elongated at the NH_2-terminus, suggesting preferential release of peptides of suboptimal length. It is not known whether CLIP(81–89) is a naturally occurring Ii fragment (*18*); if it is, it might have an editing function reminiscent of the effect of HLA-DM, which promotes dissociation of DR1-bound CLIP(81–104) and the myelin basic protein peptide MBP(90–102) but not that of stably bound influenza virus hemagglutinin peptide HA(307–319) (6).

The exact location of the effector site on DR3 to which CLIP residues 81 to 89 bind is not clear (*19*). X-ray crystallographic analysis of DR3:CLIP revealed that residues 87 to 101 of CLIP occupy the antigen-binding groove like an antigenic peptide, with Met^{91} and Met^{99} serving as primary

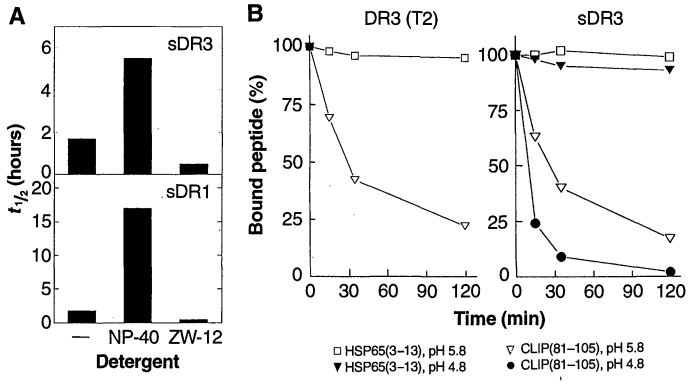

Fig. 2. Stability of CLIP:DR3 complexes generated in the absence or presence of detergent. (**A**) Effect of 0.1% Zwittergent-12 (ZW-12) and 0.5% NP-40 on the half-time of dissociation ($t_{1/2}$) of labeled CLIP(81–105) (*26*) from sDR3 (upper panel) and sDR1 (lower panel) (*27*) at pH 5.8 and 37°C. (**B**) Dissociation of CLIP(81–105) or HSP65(3–13) (*26*) from DR3 (T2) (left panel) and from recombinant sDR3 (right panel) (*27*) in the presence of 0.1% ZW-12. Dissociation was measured with an HPSEC peptide binding assay (*28*); $t_{1/2}$ values were determined from the first-order rate constants of dissociation.

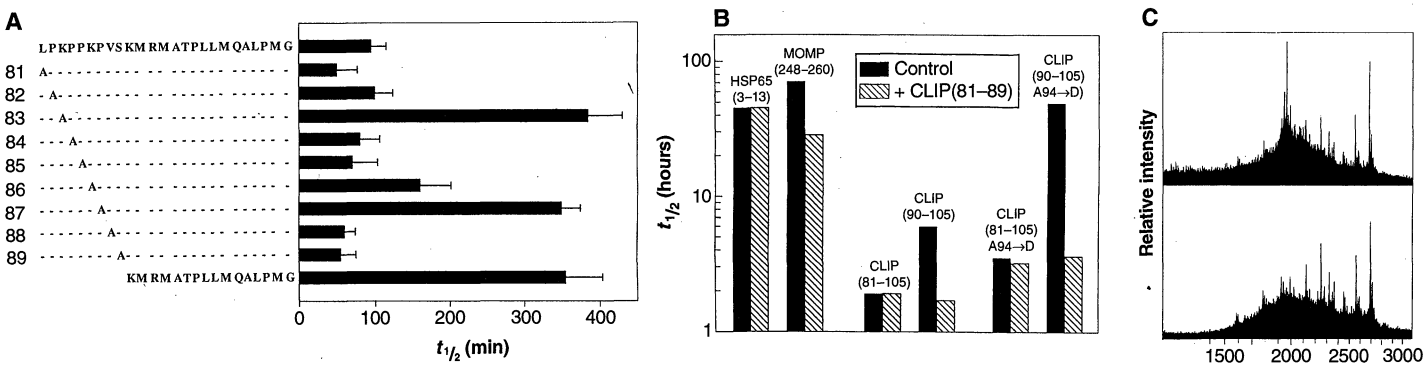

Fig. 3. Role of NH_2-terminal residues of CLIP in rapid dissociation from DR3. (**A**) Dissociation rates of alanine-scanning mutants of CLIP(81–105) bound to recombinant sDR3 were measured over 24 hours at pH 5.8 in a detergent-free system (*28*) and calculated as described in the legend to Fig. 2. Values are means ± SD from two or three individual experiments. Residue number is shown on the left. Abbreviations for amino acid residues are: A, Ala; D, Asp; G, Gly; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val. (**B**) Effect of CLIP(81–89) on the rate of dissociation of antigenic peptides and CLIP variants. Dissociation of sDR3:peptide complexes was measured in the absence (solid bars) or presence (striped bars) of 5 μM CLIP(81–89). Data are from one of two individual experiments. (**C**) Effect of CLIP(81–89) on dissociation of endogenously bound self-peptides. DR3 (5 μg) isolated from DR3-homozygous Epstein-Barr virus–transformed human B cell line COX cells was incubated for 4 hours at 37°C in the absence (upper panel) or presence (lower panel) of 5 μM CLIP(81–89). Self-peptides that remained bound to DR3 were released by acid treatment and analyzed by MALDI-MS.

anchor residues (*20*). Therefore, residues 81 to 86 must be located outside the groove. The structure of residues 81 to 86 could not be resolved in the crystal, which was generated at pH 4.5, suggesting a flexible conformation for this sequence under the latter conditions.

Given the fast rate of dissociation of CLIP at mildly acidic pH, it should be readily exchanged for exogenously added peptide. Indeed, up to 70% of the peptide-binding grooves of DR3 molecules from T2.DR3 cells, which are initially occupied predominantly by CLIP variants (Fig. 1), could be loaded with labeled HSP65(3–13) during incubation for 48 hours in vitro (Fig. 4). This binding capacity is comparable to that of "empty" sDR3 molecules, which achieve 75 to 80% occupancy. Likewise, endogenous CLIP could be readily exchanged for labeled CLIP(81–105). In contrast, only 5 to 20% of DR3 molecules isolated from DM-positive cells could bind peptides in vitro because of a high proportion of stably bound endogenous peptides. Specificity of binding was demonstrated by the failure of the DR1-restricted peptide HA(307–319) to bind to DR3 (Fig. 4).

In conclusion, our data indicate that CLIP has structural features that facilitate its own release from HLA-DR molecules at endosomal pH. Release is mediated by the NH_2-terminal residues 81 to 89, with the conserved residues Lys^{83}, Lys^{86}, and Pro^{87} being most important. The NH_2-terminal segment is able to act independently of the COOH-terminal groove-binding region of CLIP, because it is equally effective as a nonameric peptide or in the context of CLIP. Mechanistically, the NH_2-terminal region of CLIP may bind to an effector site, probably close to the peptide-binding groove of HLA-DR molecules, thereby inducing conformational changes in the groove area that result in release of CLIP as well as a subset of other peptides. HLA-DM, which has been shown to mediate dissociation of CLIP and certain peptides (*6*), might also exploit the flexibility of class II molecules and induce a conformational modulation.

Physiologically, the self-releasing properties of CLIP explain why a fraction of DR molecules from DM-negative mutant cells is associated with conventional self-peptides and why class II molecules that bind CLIP with high affinity, such as A^d, are efficiently loaded with peptides in DM-negative cells. Furthermore, DM-independent removal of CLIP may explain how loading of class II molecules is accomplished in endocytotic compartments other than the high density lysosomelike compartment MIIC (*21*), where HLA-DM has been shown to accumulate (*5*). Surface DR:CLIP complexes may also be internalized into early endosomal compartments, lose CLIP by self-release at low pH, and be loaded with processed antigenic peptides, providing a rationale for antigen presentation via recycling of class II molecules (*22*).

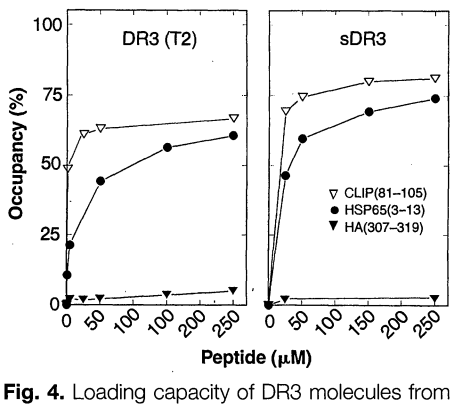

Fig. 4. Loading capacity of DR3 molecules from T2.DR3 cells (left panel) and recombinant sDR3 molecules (right panel). Binding of AMCA-labeled peptides CLIP(81–105), HSP65(3–13), and HA(307–319) (a DR1-restricted control) (*26*) at concentrations of 1 to 250 μM to purified DR3 molecules (0.2 μM) was determined after incubation for 48 hours at 37°C and pH 4.8 by the HPSEC peptide-binding assay (*28*). The percentage occupancy was calculated from the amount of DR3, the coeluting fluorescence, and the specific fluorescence of the respective peptides.

REFERENCES AND NOTES

1. P. A. Roche and P. Cresswell, *Nature* **345**, 615 (1990); O. Bakke and B. Dobberstein, *Cell* **63**, 707 (1990); P. A. Roche and P. Cresswell, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3150 (1991).
2. J. Riberdy, J. R. Newcomb, M. J. Surman, J. A. Barbosa, P. Cresswell, *Nature* **360**, 474 (1992); A. Sette *et al.*, *Science* **258**, 1801 (1992).
3. I. M. Freisewinkel, K. Schenk, N. Koch, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9703 (1993); M. E. Bijlmakers, P. Benaroch, H. L. Ploegh, *J. Exp. Med.* **180**, 623 (1994); P. Romagnoli and R. N. Germain, *ibid.*, p. 1107.
4. R. Avva and P. Cresswell, *Immunity* **1**, 763 (1994).
5. P. Morris *et al.*, *Nature* **368**, 551 (1994); S. P. Fling, B. Arp, D. Pious, *ibid.*, p. 554; L. K. Denzin, N. F. Robbins, C. Carboy-Newcomb, P. Cresswell, *Immunity* **1**, 595 (1994); L. Karlsson, A. Péléraux, R. Lindstedt, M. Liljedahl, P. A. Peterson, *Science* **266**, 1569 (1994); F. Sanderson *et al.*, *ibid.*, p. 1566.
6. V. S. Sloan *et al.*, *Nature* **375**, 802 (1995); L. K. Denzin and P. Cresswell, *Cell* **82**, 155 (1995); M. A. Sherman, D. A. Weber, P. Jensen, *Immunity* **3**, 197 (1995).
7. A. G. Brooks, P. L. Campbell, P. Reynolds, A. Gautam, J. McCluskey, *J. Immunol.* **153**, 5382 (1994); C. C. Stebbins, G. E. Loss Jr., C. G. Elias, A. Chervonsky, A. J. Sant, *J. Exp. Med.* **181**, 223 (1995).
8. D. F. Hunt *et al.*, *Science* **256**, 1817 (1992).
9. A. Sette, S. Southwood, J. Miller, E. Appella, *J. Exp. Med.* **181**, 677 (1995).
10. H. Kropshofer, H. Max, H. Kalbacher, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 403 (1993).
11. G. Malcherek, V. Gnau, G. Jung, H.-J. Rammensee, A. Melms, *J. Exp. Med.* **181**, 527 (1995); C. Lee and H. M. McConnell, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8269 (1995).
12. Rapid dissociation of CLIP(81–104) or CLIP (81–105) is not observed at neutral or basic pH or at temperatures below 20°C, explaining why it is possible to isolate MHC class II αβ:CLIP complexes from cells. These complexes are stable at 4°C during affinity-chromatographic purification and storage.
13. C. A. Nelson, S. J. Petzold, E. R. Unanue, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1227 (1993).
14. R. Urban, R. M. Chicz, J. L. Strominger, *J. Exp. Med.* **180**, 751 (1994); H. Kropshofer, A. B. Vogt, G. J. Hämmerling, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8313 (1995).
15. The sequence of murine and rat CLIP(81–105) is LPKSAKPVSQMRMATPLLMRPMSMD and LPKSAKPVSPMRMATPLLMRPLSMD, respectively.
16. MOMP(248–260) is an NH_2-terminally truncated variant of the DR3-restricted epitope from the major outer membrane protein of *Chlamydia trachomatis* (*3*).
17. S. Adams and R. E. Humphreys, *Eur. J. Immunol.* **25**, 1693 (1995).
18. It is possible that CLIP(81–89) is generated in vivo, especially in wild-type B cells expressing DR1 or DR2, which contain substantial amounts of CLIP(90–105) [R. M. Chicz *et al.*, *J. Exp. Med.* **178**, 27 (1993)].
19. DR3:CLIP(81–89) complexes are not stable, because they cannot be detected by the HPSEC binding assay at pH 5.8 and room temperature. Moreover, CLIP(81–89) does not inhibit binding of HSP65(3–13) or CLIP(81–105) to DR3 even at a 1000-fold molar excess.
20. P. Ghosh, M. Amaya, E. Mellins, D. C. Wiley, personal communication.
21. S. Amigorena, J. R. Drake, P. Webster, I. Mellman, *Nature* **369**, 113 (1994); F. Castellino and R. N. Germain, *Immunity* **2**, 73 (1995); K. A. Barnes and R. M. Mitchell, *J. Exp. Med.* **181**, 1715 (1995).
22. V. Pinet, M. Vergelli, R. Martin, O. Bakke, E. O. Long, *Nature* **375**, 603 (1995).
23. H. Kropshofer, I. Bohlinger, H. Max, H. Kalbacher, *Biochemistry* **30**, 9177 (1991).
24. DR3 molecules were isolated by affinity chromatography with the monoclonal antibody L243 [J. C. Gorga, V. Horejsi, D. R. Johnson, R. Raghupathy, J. L. Strominger, *J. Biol. Chem.* **262**, 16087 (1987)] essentially as described (*23*), with 0.1% Zwittergent-12 (Calbiochem) as the detergent in washing and elution buffers. As much detergent as possible was removed by washing solubilized DR3 molecules twice with 50 μl of H_2O in a 1-ml Centriprep ultrafiltration tube with a 5-kD cutoff (Millipore). Peptides were released by incubating DR3 molecules in 0.1% trifluoroacetic acid (pH 2.0) at 37°C for 30 min. After separation of proteins by ultrafiltration, peptides were lyophilized.
25. MALDI-MS spectra were recorded on a Finnigan Lasermat and collected by averaging the ion signals from 100 individual laser shots.
26. Peptide synthesis, purification, and specific labeling of the α amino group with 7-amino-4-methylcoumarin-3-acetic acid (AMCA) were performed as described (*23*). HSP65(3–13) corresponds to the immunodominant epitope of *Mycobacterium tuberculosis* [A. Geluk *et al.*, *J. Immunol.* **149**, 2864 (1992)], CLIP(81–105) is derived from human Ii numbered according to the p33 form of Ii [M. Strubin, C. Berte, B. Mach, *EMBO J.* **5**, 3483 (1986)], and HA(307–319) corresponds to the DR1-restricted epitope of influenza virus hemagglutinin [J. B. Rothbard *et al.*, *Cell* **52**, 515 (1988)].
27. Both sDR3 and sDR1 were obtained from Sf9 insect cells infected with baculovirus by procedures similar to those described [L. J. Stern and D. C. Wiley, *Cell* **68**, 465 (1992)].
28. DR3:peptide complexes were generated by incubation of detergent-solubilized T2.DR3 molecules (1 μM) or recombinant sDR3 (1 μM) with AMCA-labeled peptide (5 μM) for 48 hours at 37°C in 150 mM sodium phosphate at pH 4.8 or 5.8 and in the absence or presence of 0.1% ZW-12. Separation of DR3:peptide complexes from unbound peptide was achieved by HPSEC as described (*10*), with an HPSEC buffer with or without detergent. Dissociation of isolated DR3:peptide complexes was analyzed by HPSEC after various times of incubation at 37°C.

29. We thank D. C. Wiley for disclosing data before publication and R. Pipkorn for synthesis and purification of peptides. We are grateful to F. Momburg and M. Post for providing T2.DR3 cells. Supported by the Deutsche Forschungsgemeinschaft (Ha731/10-1) and the Hertie Foundation.

3 August 1995; accepted 27 September 1995