they require relatively high amounts of silicate. During times of extensive organic carbon burial in the Quaternary, they were often predominant members of the phytoplankton community, as in the case of sapropel formation in the Mediterranean (*22*) or in paleo-upwelling regions (*23*), in line with the inferred high-nutrient conditions. It may, therefore, at first sight be surprising that these diatoms did not flourish during one of the most extensive organic carbon burial events in Earth history: the Upper Cenomanian/Lower Turonian Oceanic Anoxic Event (OAE-2) (*24*). However, it has been demonstrated that during this event, owing to the stratified nature of the proto-North Atlantic ocean (*25*), there was a substantial depletion of nutrients in the photic zone, resulting in a competitive advantage of dinitrogen-fixing cyanobacteria (*26*). Breakdown of the stratification and concomitant mixing after OAE-2, because of the further opening and deepening of the Mid-Atlantic Gateway connecting the proto-North and South Atlantic Oceans, most likely resulted in higher nutrient conditions in the surface waters of the North Atlantic in the Upper Turonian. This development, initiated by plate tectonics, probably induced the evolution of open-ocean diatoms such as the *Rhizosolenia* species, which require high amounts of silicate. This type of phytoplankton has subsequently taken over the marine world and now fixes almost half of all the inorganic carbon used for photosynthesis in the ocean.

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28. We thank third parties for providing sediment and petroleum samples and diatom cultures. H. Piontkivska (University of South Carolina) is acknowledged for evolutionary rate calculations. The investigations were supported by the Dutch Technology Foundation (STW).

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www.sciencemag.org/cgi/content/full/304/5670/584/DC1
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16 February 2004; accepted 25 March 2004

An Antigenic Peptide Produced by Peptide Splicing in the Proteasome

Nathalie Vigneron,[1]* Vincent Stroobant,[1]* Jacques Chapiro,[1] Annie Ooms,[2] Gérard Degiovanni,[2] Sandra Morel,[1]† Pierre van der Bruggen,[1] Thierry Boon,[1] Benoît J. Van den Eynde[1]‡

CD8 T lymphocytes recognize peptides of 8 to 10 amino acids presented by class I molecules of the major histocompatibility complex. Here, CD8 T lymphocytes were found to recognize a nonameric peptide on melanoma cells that comprises two noncontiguous segments of melanocytic glycoprotein gp100^{PMEL17}. The production of this peptide involves the excision of four amino acids and splicing of the fragments. This process was reproduced in vitro by incubating a precursor peptide of 13 amino acids with highly purified proteasomes. Splicing appears to occur by transpeptidation involving an acyl-enzyme intermediate. Our results reveal an unanticipated aspect of the proteasome function of producing antigenic peptides.

The antigenic peptides recognized by CD8 T cells result from the degradation of intracellular proteins and correspond to small fragments of these proteins. One interesting exception was recently reported by Hanada *et al.*, who described an antigenic peptide composed of two noncontiguous segments of the parental protein (*1*). The production of this peptide implied that an intervening segment of 40 amino acids had been excised and that the products had been spliced, but the mechanism remained to be characterized. We describe here a second example of antigenic peptide produced by peptide splicing and show that the excision and splicing process is exerted by the proteasome, a multicatalytic peptidase complex that accounts for the bulk of protein degradation in the cytosol (*2*).

By stimulating blood lymphocytes of a melanoma patient with autologous tumor cells, we isolated a clone of CD8 cytolytic T lymphocytes (CTL) that recognized an antigen presented by human lymphocyte antigen (HLA)–A32 and encoded by *SILV*, the gene encoding glycoprotein gp100^{PMEL17} (Fig. 1) (*3*, *4*). This antigen is present on several melanoma cell lines. To localize the peptide recognized by this clone, named CTL 14, we transfected COS-7 cells with plasmids encoding HLA-A32 and a series of truncated gp100 sequences, and we tested the transfected cells for recognition by the CTL (Fig. 1B) (*5*). The results pointed to a 16–amino acid segment located between positions 37 and 52. When HLA-A32^{+} target cells were incubated with a series of synthetic peptides contained in this segment, none was recognized by the CTL. We considered that the peptide antigenicity might depend on a posttranslational modification, and we tried to enable this putative modification by introducing synthetic peptides inside target cells by electroporation before testing for CTL recognition. We observed that the 13–amino acid peptide RTKAWNRQLYPEW (gp100 positions 40 to 52) (*6*), which was not recognized by CTL 14 after exogenous loading on autologous Epstein-Barr virus (EBV)–transformed B cells, was recognized after electroporation into these cells (Fig. 2A). Shorter peptides lacking the N- or the C-terminal residue remained negative after electroporation.

To identify the residues that were important for antigenicity, we synthesized a series of peptides based on the sequence of the positive 13–amino acid peptide with an alanine substitution

[1]Ludwig Institute for Cancer Research and Cellular Genetics Unit, Université de Louvain, B-1200 Brussels, Belgium. [2]Laboratoire de Chirurgie Expérimentale, Université de Liège, B-4000 Liège, Belgium.

*These authors contributed equally to this work.
†Present address: GlaxoSmithKline Biologicals, Rue de l'Institut 89, 1330 Rixensart, Belgium.
‡To whom correspondence should be addressed. E-mail: benoit.vandeneynde@bru.licr.org

at each position and tested them for CTL recognition after electroporation (Fig. 2A). Whereas residues located near the N and the C terminus appeared essential for CTL recognition, the substitution of residues located in the middle region (positions 44 to 48) of the peptide affected antigenicity only marginally. Because of the recent findings of Hanada *et al.* (*1*), we considered that the modification required for antigenicity of the 13–amino acid peptide might involve the removal of those internal amino acids that appeared dispensable. We tested a series of peptides with internal deletions centered around positions 44 and 45 (Fig. 2B). We found that peptide RTKQLYPEW, the nonamer that would be produced from the 13–amino acid peptide RTKAWNRQLYPEW by removal of residues AWNR (positions 43 to 46), was efficiently recognized by CTL 14 after exogenous loading. Half-maximal lysis was achieved at a concentration of 1 nM.

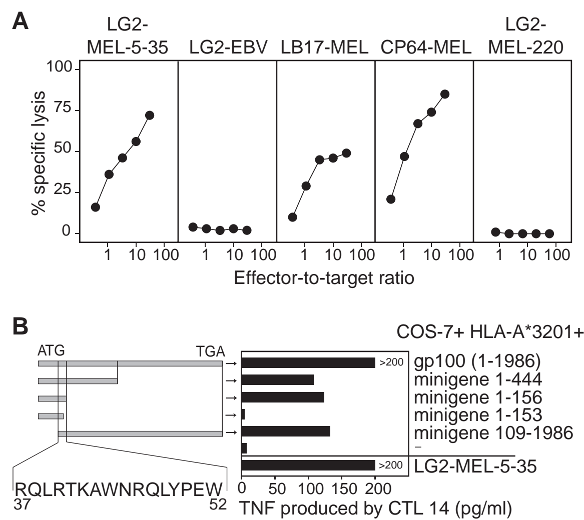

Fig. 1. Recognition by CTL 14 of an antigen derived from gp100[PMEL17]. (**A**) Lytic activity of clone CTL 14 on autologous melanoma line LG2-MEL-5-35, autologous EBV-transformed B cells LG2-EBV, allogeneic HLA-A32$^+$ gp100$^+$ melanoma lines LB17-MEL and CP64-MEL, and autologous melanoma line LG2-MEL-220, which has lost expression of gp100. (**B**) Recognition by CTL 14 of COS-7 cells transfected with the HLA-A*3201 cDNA and either the full-length gp100 cDNA or subgenic fragments of gp100. Minigenes are named according to nucleotide positions in the open reading frame. Amino acid positions are indicated below the peptide sequence.

To confirm that nonamer RTKQLYPEW was identical to the peptide presented by melanoma cells, peptides bound to HLA class I molecules purified from autologous tumor cells were eluted and separated by high-performance liquid chromatography (HPLC). The fractions of eluted peptides that stimulated the CTL were the same as those observed after running the synthetic nonamer under the same HPLC conditions (Fig. 2C).

To gain insight into the peptide-splicing mechanism, we considered the possible involvement of the proteasome, which is known to produce most antigenic peptides presented by HLA class I molecules (*2*). Two proteasome inhibitors, lactacystin and epoxomicin, prevented the recognition of target cells electroporated with the 13–amino acid peptide, indicating that proteasome activity was required for processing of this precursor peptide (fig. S1). The 13–amino acid peptide was then incubated with highly purified 20*S* proteasomes. The digests strongly stimulated production of interferon-γ (IFNγ) by CTL 14 (Fig. 3A). This was not the case when lactacystin was included in the digestion mixture. Thus, the proteasome produced the antigen in vitro, presumably by cleaving the precursor peptide and splicing two noncontiguous fragments. To confirm that the antigen produced by the proteasome was nonamer RTKQLYPEW, we separated the digest under the same HPLC conditions as before and tested the fractions with the CTL (Fig. 2C, bottom). The peptide recognized by the CTL in the digest appeared in the same fraction as the synthetic nonamer RTKQLYPEW. We then identified the peptide fragments present in the digests by HPLC coupled to mass spectrometry (MS). The observed fragments indicated cleavage after residues 42, 43, and 46 (Fig. 3B). The cleavages after 42 and 46 correspond to the boundaries of AWNR, the presumed excised fragment. Nonamer RTKQLYPEW has an expected mass-to-charge ratio (*m*/*z*) of 610.8. Only a faint signal was detected at *m*/*z* 610.8, but when this signal was further analyzed by tandem MS (MS/MS), it was identified as RTKQLYPEW on the basis of its fragmentation pattern, which was identical to that of the corresponding synthetic peptide (Fig. 3C). Thus, the proteasome appears to produce

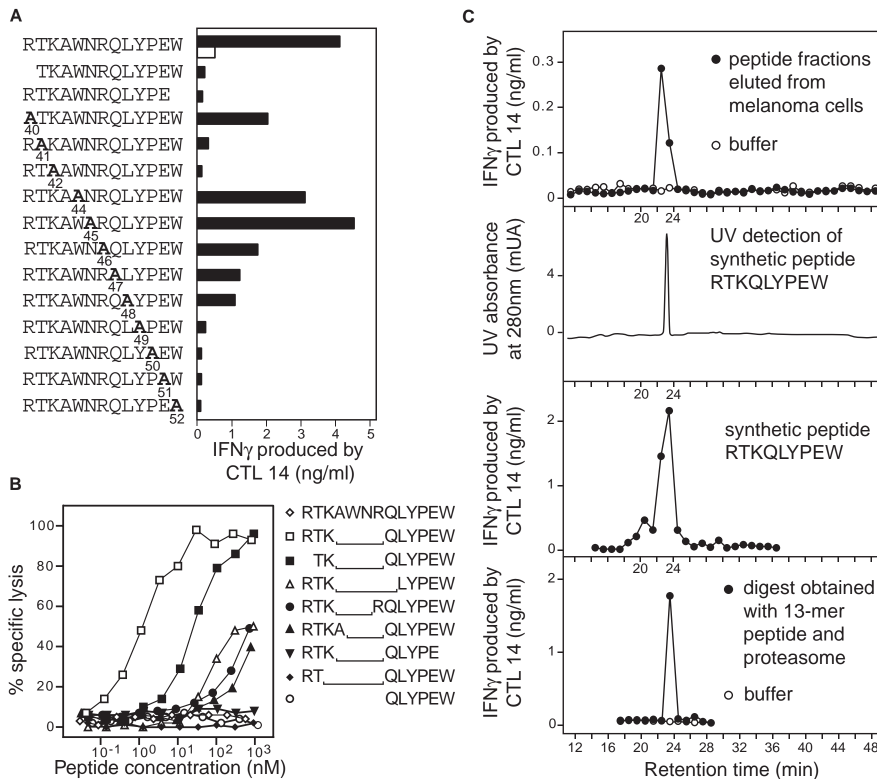

Fig. 2. Identification of the peptide recognized by CTL 14. (**A**) LG2-EBV cells were electroporated with the indicated peptides and tested for recognition by CTL 14 (black bars). As control, peptide RTKAWNRQLYPEW was pulsed onto LG2-EBV cells in the same conditions except that electroporation was omitted (white bar). The alanine substitutions are indicated with a bold A and a subscript indicating the position of the substituted residue. (**B**) Recognition of peptide RTKQLYPEW by CTL 14. ^{51}Cr-labeled LG2-EBV cells were incubated with the indicated peptides. CTL 14 was added at an effector-to-target ratio of 10. (**C**) Elution of the natural peptide recognized by CTL 14. Peptides eluted from HLA class I molecules purified from melanoma cells LG2-MEL-5-35 were separated by HPLC, and the fractions were tested for recognition by CTL 14 (top panel). To rule out contamination of the HPLC system, buffer was run on the column before the eluted samples, and the fractions were tested similarly. Synthetic peptide RTKQLYPEW (30 pmol), which was injected under the same HPLC conditions, was monitored by ultraviolet (UV) absorption, and the fractions were tested for CTL recognition (middle panels). A digest obtained after a 120-min incubation of 20*S* proteasomes with peptide RTKAWNRQLYPEW was separated and tested similarly (bottom panel). mUA, milli-units of absorbance; 13-mer peptide, 13–amino acid peptide.

peptide RTKQLYPEW by excision and splicing, albeit at a very low efficiency. A comparison of the level of CTL activation obtained with the digests or with a titration curve of the synthetic nonamer provided a rough estimate of one spliced peptide produced from about 10^4 molecules of precursor peptide. The extreme sensitivity of CTL, which can recognize target cells presenting fewer than 10 molecules of antigenic peptide, probably explains why such a low-efficiency process leads to lysis of melanoma cells by CTL 14 (*7*, *8*).

Presumably, the proteasome produces the antigenic peptide by cleaving the precursor peptide and catalyzing the formation of a peptide bond between two distant fragments. Because the production of the antigenic peptide occurs in vitro in the absence of exogenous adenosine 5′-triphosphate, the energy required to form the new peptide bond must be recovered from one of the bonds cleaved by the proteasome. This could occur by transpeptidation as follows: Cleavage by the proteasome is known to occur by nucleophilic attack of the peptide bond by the catalytic threonine, resulting in the formation of an acyl-enzyme intermediate in which a peptide fragment is attached by an ester bond to the catalytic threonine (*9*). Thus, the observed cleavage of precursor RTKAWNRQLYPEW after residue 42 produces an intermediate that comprises fragment RTK attached to the catalytic threonine (Fig. 4A). Normally, this intermediate is rapidly hydrolyzed. However, inside the confined catalytic chamber of the proteasome, the intermediate is surrounded by peptide fragments, such as QLYPEW resulting from cleavage after residue 46. The N terminus of this fragment could compete with water molecules and occasionally make a nucleophilic attack of the ester bond of the intermediate, thereby forming a new peptide bond and producing the antigenic peptide RTKQLYPEW (Fig. 4A).

To test this model, we first incubated proteasomes with two distinct peptides each containing a different portion of the precursor peptide, RTKAWNR and AWNRQLYPEW. The digests were recognized by CTL 14 almost as efficiently as were the digests obtained with the 13–amino acid precursor (Fig. 4B). We then incubated proteasomes with peptides RTK and QLYPEW and observed that the antigenic peptide was not produced (Fig. 4B). This confirmed that splicing required recycling of the energy of a peptide bond. We also observed that the antigenic peptide was produced after incubation with RTKAWNR and QLYPEW but not with RTK and AWNRQLYPEW (Fig. 4B). This indicated that the energy of the new peptide bond was recycled from the bond between K^{42} and A^{43} and not from that between R^{46} and Q^{47}. This supported the model of formation of an acyl-enzyme intermediate between RTK and the proteasome, which is attacked by fragment QLYPEW. That the N terminus of the latter fragment was indeed responsible for this nucleophilic attack was supported by the fact that its N-α-acetylation completely prevented the production of the antigenic peptide (Fig. 4B).

This model predicts that splicing does not depend on a particular sequence motif, but could occur with any fragment produced by the proteasome. We therefore examined whether the digests contained other spliced peptides. The other cleavage site of the 13–amino acid peptide occurred after residue 43, producing fragment RTKA (Fig. 3B). We searched the digests for the presence of peptide RTKAQLYPEW, which would result from splicing of RTKA with QLYPEW. A signal detected at the expected *m*/*z* for this peptide was identified by MS/MS as RTKAQLYPEW (Fig. 3D).

Protein splicing has been described in unicellular organisms as the autocatalytic excision of segments named inteins, which are up to several hundred amino acids in length (*10*). The peptide splicing described here differs in several respects. It affects much smaller segments and is not autocat-

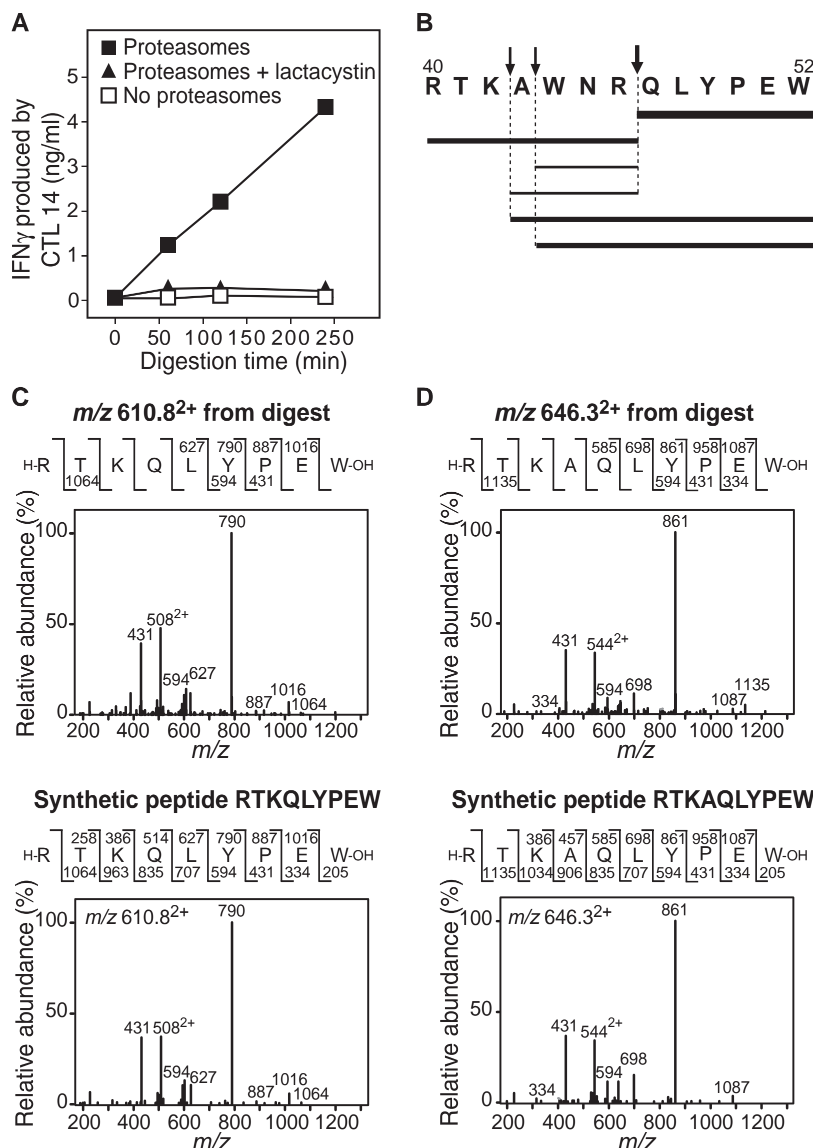

Fig. 3. Role of 20*S* proteasomes in peptide splicing. (**A**) Production of the spliced peptide by incubation of 20*S* proteasomes in vitro with the 13–amino acid precursor RTKAWNRQLYPEW. Digests were pulsed onto LG2-MEL-220 target cells and tested for recognition by CTL 14. (**B**) Main peptide fragments detected by mass spectrometry after a 120-min digestion of the 13–amino acid precursor by 20*S* proteasomes. The lines indicate the various fragments observed, and their thickness is related to the amount of peptide observed. The experimental conditions did not allow the detection of fragments RTK and RTKA. (**C**) MS/MS fragmentation spectrum of the doubly charged ion with *m*/*z* 610.8^{2+} observed in the digest obtained after a 60-min incubation of 20*S* proteasomes with 13–amino acid peptide RTKAWNRQLYPEW (top), and fragmentation spectrum of synthetic nonamer RTKQLYPEW (bottom). The fragments that were detected are indicated above the peptide sequence for N-terminal ions and below for C-terminal ions. (**D**) MS/MS fragmentation spectrum of the doubly charged ion with *m*/*z* 646.3^{2+} observed in the same digest as in (C) (top) and fragmentation spectrum of synthetic decamer RTKAQLYPEW (bottom).

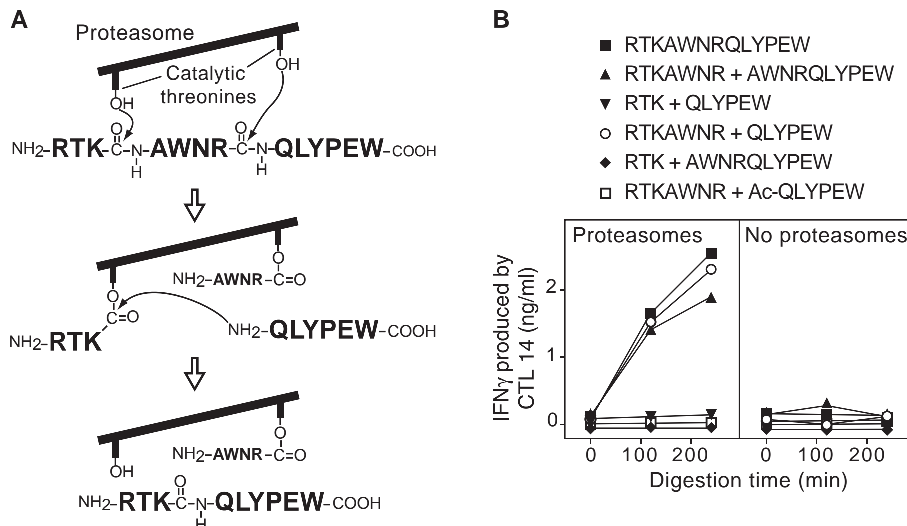

Fig. 4. Mechanism of peptide splicing. (**A**) Model of the peptide splicing reaction. (**B**) Various synthetic peptides were combined in a pairwise manner and incubated with 20*S* proteasomes. Digests were tested for recognition by CTL 14. Mass spectrometry confirmed the presence of RTKQLYPEW in the digests recognized by the CTL and its absence in the others. Ac-QLYPEW, N-α-acetylated peptide QLYPEW.

alytic. Rather, it is catalyzed by the proteasome and therefore takes place during protein degradation.

Peptide identification efforts have provided many examples of antigenic peptides that do not simply correspond to fragments of conventional proteins, but rather result from aberrant transcription, incomplete splicing, translation of alternative or cryptic open reading frames, or post-translational modifications (*11–16*). Peptide splicing is another mechanism that increases the diversity of antigenic peptides presented to T cells. It represents a new aspect of the proteasome function in antigen processing.

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17. We thank P. Coulie for suggestions, N. Demotte for help with peptide electroporation, S. Claverol for evaluating the purity of proteasome preparations, and E. Van Schaftingen, L. Hue, D. Godelaine, J. Berthet, and E. Warren for critical reading of the manuscript. N.V. and J.C. were supported by a Télévie fellowship from the Fonds National de la Recherche Scientifique (FNRS), Belgium. This work was supported by grants from the FNRS and the Fédération Belge contre le Cancer (Belgium).

Supporting Online Material
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12 January 2004; accepted 25 February 2004
Published online 4 March 2004;
10.1126/science.1095522
Include this information when citing this paper.

CD8αα-Mediated Survival and Differentiation of CD8 Memory T Cell Precursors

Loui T. Madakamutil,[1] Urs Christen,[1] Christopher J. Lena,[1] Yiran Wang-Zhu,[1] Antoine Attinger,[1] Monisha Sundarrajan,[1] Wilfried Ellmeier,[2]* Matthias G. von Herrath,[1] Peter Jensen,[3] Dan R. Littman,[2] Hilde Cheroutre[1]†

Memory T cells are long-lived antigen-experienced T cells that are generally accepted to be direct descendants of proliferating primary effector cells. However, the factors that permit selective survival of these T cells are not well established. We show that homodimeric α chains of the CD8 molecule (CD8αα) are transiently induced on a selected subset of CD8αβ$^+$ T cells upon antigenic stimulation. These CD8αα molecules promote the survival and differentiation of activated lymphocytes into memory CD8 T cells. Thus, memory precursors can be identified among primary effector cells and are selected for survival and differentiation by CD8αα.

The majority of T cells responding during a primary immune response subsequently undergo programmed cell death. However, a fraction of activated T cells survive and differentiate into long-lived memory T cells (*1*). What mechanisms mediate the selective survival of these cells? To address this question, we first must identify those effector T lymphocytes that will differentiate into memory cells.

The homotypic form of CD8 that uses the α chain of the molecule (CD8αα) appears to serve functions that are distinct from those of the T cell receptor (TCR) coreceptors CD4 and CD8αβ (*2–4*). Immature thymocytes can induce CD8αα upon strong TCR stimulation (*5–7*), and in mice (*4*, *8–11*) and humans (*12*, *13*), CD8αα is expressed on distinct T cell subsets that constitutively display a memory phenotype. In light of these characteristics, we hypothesized that CD8αα might have functional relevance in specifying T cell memory fate.

We recently showed that the thymic leukemia antigen TL, a nonclassical major histocompatibility complex (MHC) class I molecule, is a unique ligand for CD8αα, with TL tetramers binding specifically to

[1]La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121, USA. [2]Howard Hughes Medical Institute and Skirball Institute of Biomedical Science, New York University School of Medicine, New York, NY 10016, USA. [3]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA.

*Present address: Institute of Immunology, University of Vienna, Brunner Strasse 59, 1235 Vienna, Austria. †To whom correspondence should be addressed. E-mail: hilde@liai.org