induce any changes in recipient mice. It seemed unlikely that the adoptive transfer of contaminating granulocytes was responsible for the granulocyte abnormality, because mice injected with mutant spleen cells containing >90% granulocytes did not develop an abnormal phenotype in any cell lineage. However, the expression of IL-2Rβ on myeloid cells necessitates cautious interpretation of these data; we cannot yet exclude the possibility that receptor deficiency may play some direct role in the observed myeloid disorder.

Taken together, our results suggest that IL-2Rβ, in addition to its known role as a mediator of activation signals for T cell proliferation, is also a central component of the regulatory system in T cells. It appears that a complete lack of signaling through the IL-2Rβ chain can result in deregulation of these lymphocytes and consequent loss of homeostasis and autoimmunity.

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30. We thank V. A. Wallace, J. Penninger, and K. Kawai for valuable suggestions with experiments; B. W. Zanke for critical reading of the manuscript; L. Prevec for providing VSV; and M. Trevisan, A. Shahinian, and J. Potter for technical assistance. Supported by grants from the Medical Research Council of Canada, the National Cancer Institute of Canada, and the National Science and Engineering Research Council of Canada. H.S. was supported by a postdoctoral fellowship from the Cancer Research Institute; T.M.K. was supported by the Swiss National Science Foundation; and T.M. and P.S.O. were supported by the Medical Research Council of Canada.

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Identification of a Graft Versus Host Disease–Associated Human Minor Histocompatibility Antigen

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Minor histocompatibility antigen disparities between human leukocyte antigen (HLA)–matched bone marrow donors and recipients are a major risk factor for graft versus host disease (GVHD). An HLA-A2.1–restricted cytotoxic T cell clone that recognized the minor histocompatibility antigen HA-2 was previously isolated from a patient with severe GVHD after HLA-identical bone marrow transplantation. The HLA-A2.1–bound peptide representing HA-2 has now been identified. This peptide appears to originate from a member of the non–filament-forming class I myosin family. Because HA-2 has a phenotype frequency of 95 percent in the HLA-A2.1–positive population, it is a candidate for immunotherapeutic intervention in bone marrow transplantation.

In the 1970s, human bone marrow transplantation (BMT) became available as a therapy for severe aplastic anemia, leukemia, and immune deficiency disease (*1*). The long-term results of allogeneic BMT have greatly improved for a variety of reasons, including the participation of HLA-matched siblings as marrow donors, advancements in pretransplant chemoradiotherapy, the prophylactic use of potent immunosuppressive drugs, and better antibiotics and isolation procedures. Nonetheless, the selection of major histocompatibility complex (MHC)–identical donors and recipients does not guarantee avoidance of GVHD, nor does it ensure disease-free survival, even when the donor and the recipient are closely related (*2*). Up to 80% of cases of GVHD result from allogeneic BMT between unrelated HLA-matched adults (*3*, *4*). Disparities in minor histocompatibility antigens (mHags) between the donor and the recipient constitute a significant risk for graft failure or GVHD. These conditions necessitate lifelong pharmacological immunosuppression of organ and BMT recipients (*3*, *5*, *6*).

Cytotoxic T lymphocytes (CTLs) that are specific for host mHags have been detected in patients who develop GVHD after BMT from donors who are genotypically HLA-identical (*7*). Immunogenetic analyses with CTL clones have identified five non–sex-linked mHags, designated HA-1 through -5, that are recognized in a classical MHC-restricted fashion (*8*) and are products of single genes that segregate in a Mendelian fashion (*9*). In a prospective study, mismatching of these mHags significantly correlated with GVHD (*6*). These mHags show defined phenotype frequencies (*8*) and tissue distribution patterns (*10*). HA-1 and HA-2 are uniquely expressed on hematopoietic-derived cells, including leukemic cells (*11*), whereas HA-3 and HA-4 are present on other cell types as well (*10*).

Two unusual mHags have been charac-

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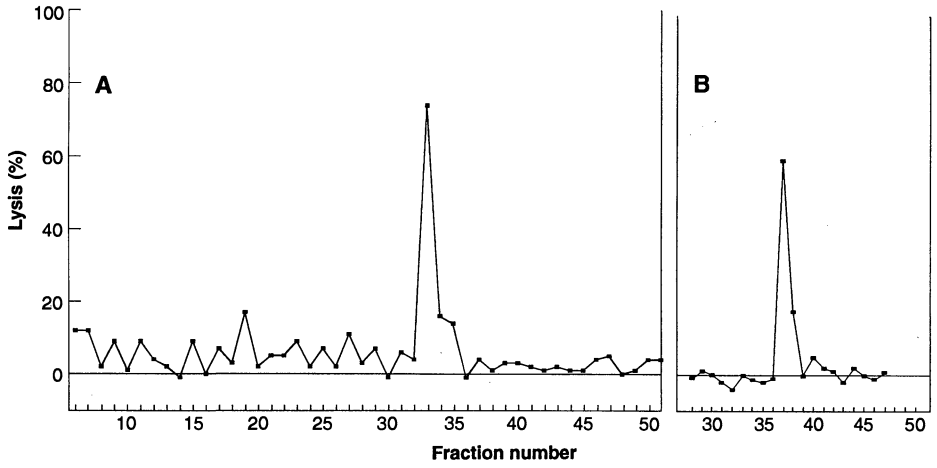

Fig. 1. HPLC fractionation and ^{51}Cr release assay of HLA-A2.1–bound peptides. Peptides were eluted out of HLA-A2.1 molecules as described (*14*) and were fractionated by reversed-phase HPLC. Buffer A was 0.1% trifluoroacetic acid (TFA); buffer B was 0.1% TFA in acetonitrile. (**A**) The gradient consisted of 100% buffer A (0 to 20 min), 0 to 12% buffer B (20 to 25 min), and 12 to 50% buffer B (25 to 80 min) at a flow rate of 100 μl/min. Fractions of 100 μl were collected. (**B**) The gradient consisted of 100% buffer A (0 to 29 min), 0 to 22% buffer B (29 to 34 min), 22% buffer B (34 to 39 min), 22 to 27.9% buffer B (39 to 98 min), 27.9% buffer B (98 to 103 min), and 27.9 to 100% buffer B (103 to 108 min) at a flow rate of 100 μl/min. Fractions of 100 μl were collected. Peptide fractions (25 μl) were diluted [1:50 in (A) and 1:30 in (B)] in Hanks' balanced salt solution (HBSS) containing 50 mM Hepes and were incubated with 2500 ^{51}Cr-labeled T2 cells in a total volume of 50 μl for 30 min before incubation with CTL at an effector:target ratio of 18:1 in (A) and 30:1 in (B). Background lysis of T2 by CTL clone 5H17 in the absence of peptide fractions was 5% in (A) and 2% in (B). Spontaneous release of T2 incubated with peptide fractions and in the absence of clone 5H17 never exceeded 7% in (A) and 5% in (B). Lysis of HA-2–positive EBV-BLCLs by clone 5H17 was 83% in (A) and 73% in (B).

terized at the molecular level: H-3, which depends on allelic forms of β_2-microglobulin associated with class I MHC molecules (*12*), and the mitochondrially encoded, maternally transmitted antigen presented by Hmt molecules (*13*). However, although some conventional murine and human mHags have been shown to be peptides presented by MHC molecules (*14*, *15*), the exact amino acid sequence of these peptides and the identification of the proteins from which they originate have not been reported. Here, we report the identification by tandem mass spectrometry of a peptide that represents the HLA-A2.1–restricted HA-2 epitope.

To isolate HA-2, we used affinity chromatography to purify HLA-A2.1 molecules from Epstein-Barr virus transformed B lymphocytes (EBV-BLCLs) expressing HA-2. The HLA-A2.1–bound peptides were isolated by acid treatment and 10-kD filtration (*13*). These low molecular mass molecules were fractionated by reversed-phase high-performance liquid chromatography (HPLC), and individual fractions were analyzed for HA-2–sensitizing activity by incubation with the HA-2– negative, HLA-A2.1–positive lymphoblastoid cell line T2 in a ^{51}Cr release assay. One fraction (fraction 33) sensitized T2 for lysis by the HA-2–specific CTL clone 5H17 (*16*) (Fig. 1A).

When fraction 33 was rechromatographed with a shallower gradient, HA-2–sensitizing activity was observed in fractions 37 and 38 (Fig. 1B). However, as assessed by microcapillary HPLC–electrospray ionization tandem mass spectrometry, the latter fractions still contained more than 100 different HLA-A2–binding peptides (*17*). To determine which of the peptides was responsible for the HA-2–sensitizing activity, we rechromatographed fraction 37 by microcapillary HPLC. The effluent was split with an on-line splitter (*18*) and was directed simultaneously into the mass spectrometer and into a 96-well microtiter plate for a subsequent ^{51}Cr release assay. In this way, mass spectrometry data on individual peptides could be correlated with epitope recon-

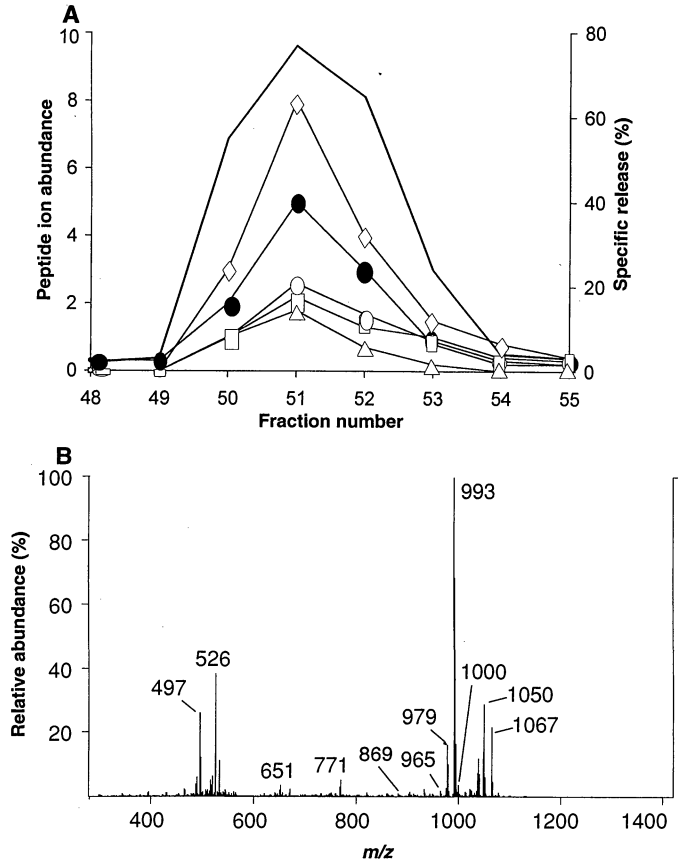

Fig. 2. Determination of candidate peptides by mass spectrometry combined with ^{51}Cr release assay. Second-dimension fraction 37 (from Fig. 1B) was separated with an on-line microcapillary effluent splitter (*19*). A C_{18} microcapillary HPLC column (100 μm by 25 cm) was butt-connected with a zero dead volume union (Valco, Phoenix, Arizona) to two capillaries of different lengths and interior diameters (25 and 40 μm; Polymicro Technologies, Houston, Texas), and peptides were eluted with a 34-min gradient of 0 to 60% acetonitrile. The 25-μm capillary deposited one-sixth of the material into microtiter plate wells (10-s fractions) containing 150 μl of RPMI 1640 with 10% human serum culture medium for CTL analysis. A 50-μl portion of the contents of each well was incubated with 2500 T2 cells in a final volume of 100 μl for 1 hour at 37°C, and 50 μl of CTL was added to yield an effector:target ratio of 40:1. The 40-μm capillary directed the remaining five-sixths of the material into the electrospray ionization source, and the mass spectra of the peptides deposited into each well were recorded on a triple-quadrupole mass spectrometer (Finnigan-MAT, San Jose, California). (**A**) Epitope reconstituting activity is measured as percent specific release (solid line). Peptide ion abundance is shown for peptides with *m/z* values of 651 (◇), 869 (△), 965 (□), 979 (○), and 1000 (●). Full scale is 10^5 counts except for 979, which is 10^6 counts. (**B**) Summation of mass spectra recorded on peptides deposited in well 51. Full scale is 1.4×10^7 counts.

stitution activity. Figure 2A shows a single peak of HA-2–sensitizing activity in wells 50 to 53. Of the many peptides present in these wells (Fig. 2B), the relative ion abundance profile of five [with mass-to-charge ratios (m/z) of 651, 869, 965, 979, and 1000] matched the activity profile of the HA-2–specific CTL–sensitizing activity (Fig. 2A). Collision-activated dissociation (CAD) analysis performed for the species with m/z of 979 revealed the existence of two different peptides, YXGEVXVSV and SXDFGTXQV (*19*) (Fig. 3, A and B). The X represents L or I, which cannot be distinguished by mass spectrometry under these conditions. We next made synthetic peptide mixtures with an equimolar mixture of L and I in place of X and assayed them for HA-2–specific CTL-sensitizing activity. Only incubation with peptide mixture YXGEVXVSV resulted in lysis of T2 (*17, 20*).

To identify the naturally processed peptide epitope, we synthesized four peptides with I or L at positions 2 and 6. Microcapillary HPLC coelution studies revealed that three of these synthetic peptides (YIGEVLVSV, YLGEVLVSV, and YLGEVIVSV) coeluted with the naturally processed peptide, whereas peptide YIGEVIVSV did not (*17*). These three peptides could not be distinguished from one another or from the naturally processed peptide under several different HPLC elution conditions. All three peptides sensitized the T2 cell line for lysis by clone 5H17 (Fig. 4A). Peptide YIGEVLVSV sensitized targets for half-maximal lysis at a concentration of 40 pM, whereas the sensitizing concentrations for peptides YLGEVLVSV and YLGEVIVSV were substantially higher (1.5 and 2.25 nM, respectively). These concentrations were within the range of 10 pM to 50 nM established for other naturally processed epitopes (*18, 21*). Panel analysis indicates that clone 5H13 is an independently derived CTL that also recognizes HA-2 but differs slightly from clone 5H17 in its fine specificity of antigen recognition (*8, 22*). Clone 5H13 also recognized all three peptide variants (Fig. 4B). Although the concentration of peptides necessary to give half-maximal epitope reconstitution was 5 to 10 times higher than for clone 5H17, peptide YIGEVLVSV still sensitized at one-hundredth the concentration of the other two. Thus, despite their fine specificity differences, both HA-2–specific CTL clones recognized the same peptide epitope.

Binding studies with these three peptides showed that peptide YIGEVLVSV also had the highest binding affinity for HLA-A2.1. The concentration of YIGEVLVSV that inhibited the binding of the iodinated standard peptide to purified HLA-A2.1 by 50% (IC_{50}) was 6.7 nM, whereas the IC_{50} values for YLGEVLVSV and YLGEVIVSV were 17 and 27 nM, respectively (Fig. 5). These

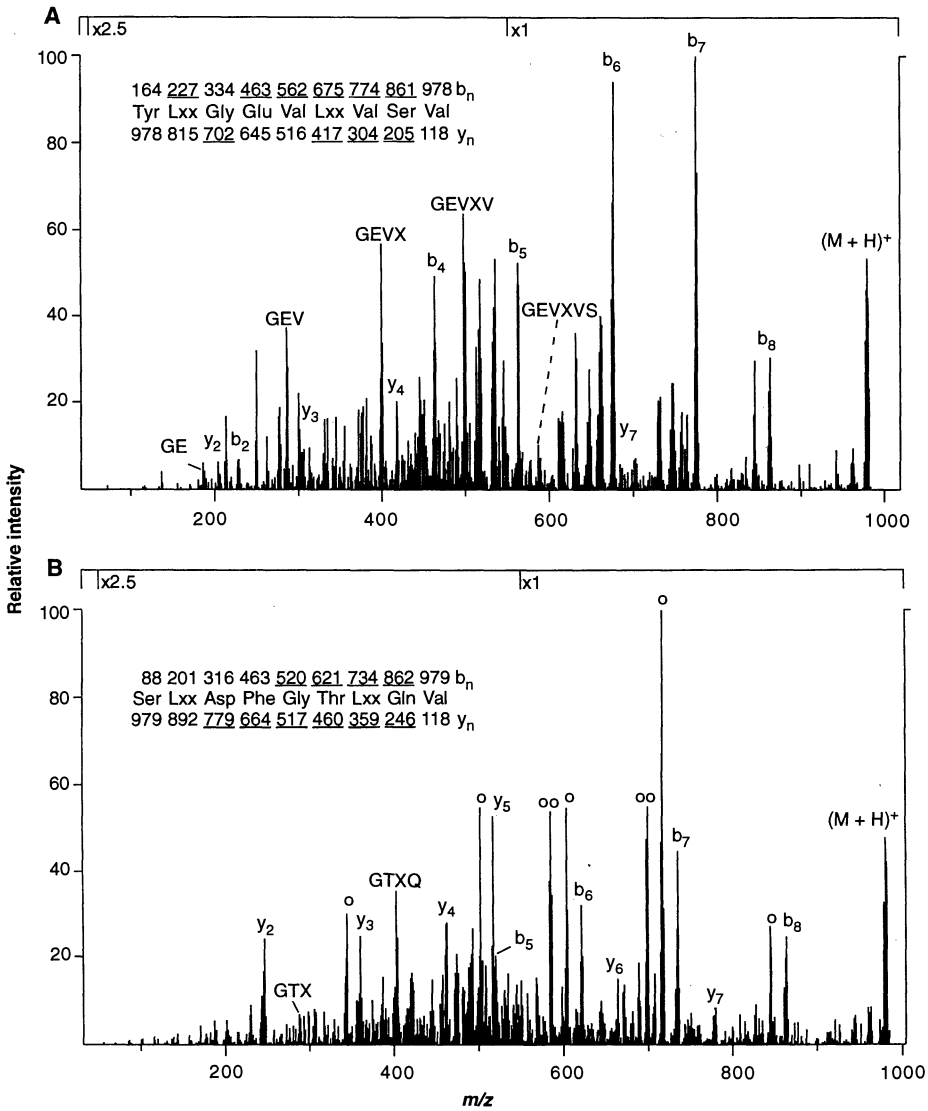

Fig. 3. CAD mass spectra of candidate peptide $(M + H)^+$ ions with m/z of ~979. Spectra were recorded on a triple-quadrupole mass spectrometer (Finnigan-MAT) operating with a 2–mass unit window in quadrupole 1. Predicted masses for fragment ions of types b and y (*30*) are shown, respectively, above and below the deduced amino acid sequence. Ions observed in the spectrum are underlined. Subtraction of m/z values for any two fragments that differ by a single amino acid generates a value that specifies the mass and thus the identity of the extra residue in the larger fragment. Lxx represents Ile or Leu, which have identical mass and cannot be differentiated on the mass spectrometer. (**A**) Peptide YXGEVXVSV (actual m/z of 978). (**B**) Peptide SXDFGTXQV (actual m/z of 979). O indicates ions that represent single and multiple losses of water.

values place these peptides among the highest-affinity naturally processed peptides that have been identified to date (*23*). However, although there is less than a fourfold difference in binding affinities among these three peptides, peptide YIGEVLVSV sensitizes target cells for recognition by clones 5H17 and 5H13 at one-fiftieth to one-hundredth the concentration of the other two. This finding indicates that peptide YIGEVLVSV is recognized with the highest affinity by the T cell receptors of both of these CTL clones, and that it is the actual HA-2 epitope.

To determine the quantity of HA-2 peptide present on EBV-BLCLs, we compared a positive HPLC fraction with a known amount of synthetic peptide on the mass spectrometer. Assuming an overall purification yield of 12% (*18*), we estimated that 260 HA-2 peptide–HLA-A2.1 complexes were expressed per cell. This number is similar to values determined for other sequenced T cell epitopes (*18, 21*), and it indicates that HA-2 is moderately abundant compared with other naturally processed peptides (*18, 24*).

A search of DNA and protein sequence databases identified six distinct coding sequences that match peptide YIGEVLVSV at eight of nine residues, and an additional seven that match at seven of nine residues. All of these sequences are derived from non-

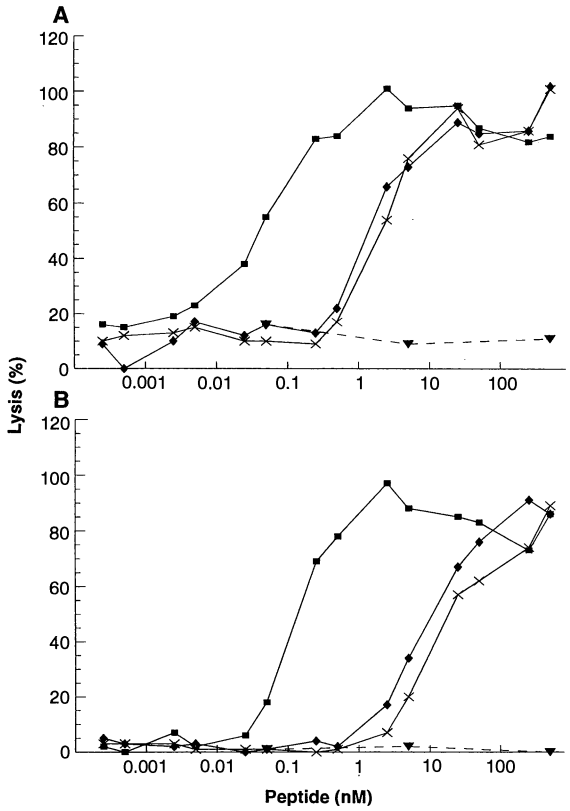

Fig. 4. Ability of synthetic peptides to reconstitute recognition by HA-2 CTL clones. Dose response curves of peptides YIGEVLVSV (■), YLGEVLVSV (◆), YLGEVIVSV (×), and SXDFGTXQV (▼) are shown for (**A**) CTL clone 5H17 and (**B**) CTL clone 5H13. Peptides were incubated with 2500 T2 cells in a total volume of 50 μl of HBSS containing 50 mM Hepes for 30 min at 37°C. In (A), 100 μl of clone 5H17 was added to yield an effector:target ratio of 26:1; in (B), 100 μl of clone 5H13 was added to yield an effector:target ratio of 7:1. Background lysis of T2 in the absence of peptide was 12% by clone 5H17 in (A) and 1% by clone 5H13 in (B). Spontaneous release of T2 incubated with peptide never exceeded 5%. Lysis of HA-2–positive EBV BLCLs was 86% by clone 5H17 in (A) and 93% by clone 5H13 in (B).

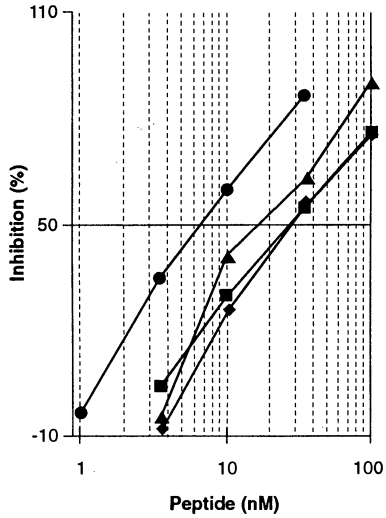

Fig. 5. Binding of synthetic peptides to purified HLA-A2.1. HPLC-purified peptides were assayed for the ability to inhibit the binding of the iodinated hepatitis B core antigen peptide FLPSDYFPSV to purified HLA-A2.1 molecules (*23*). ●, YIGEVLVSV; ▲, YLGEVLVSV; ■, YLGEVIVSV; and ◆, the influenza M1 protein antigen GILGFVFTL. All data points are the average of at least two independent experiments.

filamentous class I myosins, a large family of proteins that are involved in cell locomotion and organelle transport (*25*, *26*). Most mismatches to the HA-2 sequence occurred at peptide position 4. Nonetheless, at least two class I myosin sequences contain a glutamic acid at this position. The closest known human sequence corresponds to residues 51 to 59 from unconventional myosin IC (YIGSVLISV), which is also the only completely sequenced class I myosin gene (*27*). However, although different cell types appear to express multiple class I myosins simultaneously, tissue-restricted expression of class I myosins has also been reported (*26*, *28*). Because the total number of family members is still not known, the HA-2 peptide epitope is most likely derived from an as yet uncharacterized human class I myosin protein.

Virtually all of the known class I myosin sequences, regardless of their species of origin, show identity at six or more of the nine positions within the sequence that corresponds to the HA-2 peptide, and these sequences also contain an isoleucine at position 2 and a valine at position 9. These latter residues represent the most important elements of the HLA-A2.1–binding motif (*23*, *29*). Thus, the basis for differential expression of the mHag HA-2 within the human population may be allelic polymorphism that results in presentation of homologous but nonidentical peptides from this region by HLA-A2.1 molecules, or in the failure to present a peptide that has lost one of the motif residues. However, the large amount of HA-2–related sequence conservation among different class I myosins from the same species also suggests that several peptides related to HA-2 may be expressed in association with HLA-A2.1 in a single individual and may be cross-reactively recognized by HA-2–specific T cells. Indeed, the human class IC myosin–derived peptide YIGSVLISV was able to sensitize T2 cells for lysis by clones 5H17 and 5H13 and could induce 50% lysis at concentrations of 5 to 50 nM (*17*). Because these peptides could also exert a profound tolerogenic effect on the HA-2–specific T cell repertoire, an alternative hypothesis is that a polymorphism in the class I antigen processing system results in a failure to express all or many members of this set of peptides in HA-2–negative individuals. Finally, because HA-2 is only presented by hematopoietic cells, either the expression of its unknown class I myosin source protein is tissue-specific or HA-2 is only presented as a consequence of tissue-specific processing.

Until now, information on mHags has been scarce. Although the physiological function of mHags is still unknown, their pivotal role in organ transplantation in general, and in BMT in particular, is undeniable. The availability of the mHag peptide sequence may allow in vivo modification of GVHD–related T cell responses. Because the mHag HA-2 is expressed on cells of the hematopoietic lineage, including leukemic cells, it is a candidate for immunotherapy for leukemia before BMT.

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14. The HA-2–specific CTL clone 5H17 originated from a female patient who underwent BMT for severe aplastic anemia. The pretransplant conditioning regime consisted of total lymphoid irradiation and cyclophosphamide. The patient was grafted with non–T cell-depleted bone marrow from her HLA-identical

father. She developed severe acute GVHD (grade III) followed by extensive chronic GVHD. The HA-2–specific CTL clone was generated from post-BMT peripheral blood lymphocytes according to the protocol described earlier [E. Goulmy, in *Transplantation Reviews, Vol. 2*, J. Morris and N. L. Tilney, Eds. (Saunders, Philadelphia, 1988), pp. 29–53].
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19. Single-letter abbreviations for the amino acid residues are as follows: D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; L, Leu; P, Pro; Q, Gln; S, Ser; T, Thr; V, Val; and Y, Tyr.
20. Peptide mixtures YXGEVXVSV and SXDFGTXQV were tested in several concentrations against clones 5H17 and 5H13. In addition to T2, an HA-2–negative, HLA-A2.1–positive EBV-BLCL was used to present the peptide mixture.
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Growth of *Prochlorococcus*, a Photosynthetic Prokaryote, in the Equatorial Pacific Ocean

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The cell cycle of *Prochlorococcus*, a prokaryote that accounts for a sizable fraction of the photosynthetic biomass in the eastern equatorial Pacific, progressed in phase with the daily light cycle. DNA replication occurred in the afternoon and cell division occurred at night. Growth rates were maximal (about one doubling per day) at 30 meters and decreased toward the surface and the bottom of the ocean. Estimated *Prochlorococcus* production varied between 174 and 498 milligrams of carbon per square meter per day and accounted for 5 to 19 percent of total gross primary production at the equator. Because *Prochlorococcus* multiplies close to its maximum possible rate, it is probably not severely nutrient-limited in this region of the oceans.

Assessment of microbial population growth rate in aquatic systems is difficult because ubiquitous grazers remove cells as quickly as they are produced. If the cell cycle of a population is in phase with the daily light cycle, cell division rates can be estimated from changes in the fractions of cells in each cell cycle stage (*1*). This approach does not require sample incubation, which is always a potential source of artifacts. We used cell cycle fractions to investigate division rates of the phytoplanktonic prokaryote *Prochlorococcus* (*2*) in the equatorial Pacific at 140°W (Table 1). Maximum cell abundances of *Prochlorococcus* occurred in general in the top 45 m and varied in a near-threefold range from 60,000 to 170,000 cells ml^{-1} (Fig. 1). Below 45 m, *Prochlorococcus* decreased in abundance but was present down to 150 m (1 April, Fig. 1). Flow cytometric DNA histograms displayed a single peak composed of cells in the G_1 phase of the cell cycle (*3*) during the late hours of night and in the early morning (Fig. 2). Around 1400 (arrowhead, Fig. 2), a distinct population of cells began to enter the S phase at 30 m and below, while cells in the surface layer (0 to 15 m) remained in G_1. By 1700, a large fraction of cells in the deeper samples had finished genomic DNA replication and entered the G_2 phase. Meanwhile, in the surface layer (0 and 15 m), cells had entered the S phase. By 2300, cells at all depths had divided and reentered the G_1 phase. This pattern was very closely reproduced in each of the five diel cycles (Table 1) sampled. Analysis of the fraction of cells in the various cell cycle phases as a function of time (Fig. 3) further reveals the extremely tight cell synchrony induced by the daily light cycle. Two independent indexes of this synchrony are the very large fraction of cells found in S in the afternoon (up to 90%) and the brevity of the S and G_2 phases, which last 4 and 2 hours, respectively [as computed according to Carpenter and Chang (*1*)]. Such synchronous cell cycling, as well as the timing of cell division early in the night, differs from other reports of phasing for oceanic phytoplankton: Dinoflagellates usually divide late in the night (*4*), whereas the cyanobacterium *Synechococcus*, a close relative of *Prochlorococcus* (*5*), divides throughout the whole daylight period (*6*).

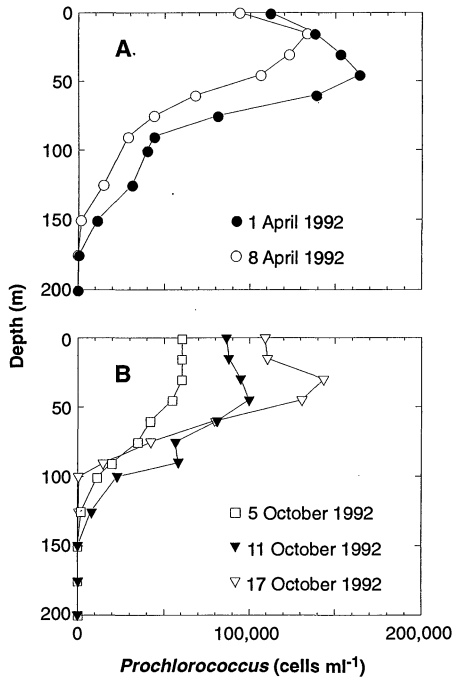

Fig. 1. Vertical profiles of *Prochlorococcus* cell concentrations at the equator (140°W) for five diel cycles sampled during the U.S. Joint Global Ocean Flux Study equatorial Pacific study in April (**A**) and October (**B**) 1992. Samples were preserved with 0.1% glutaraldehyde, frozen, and kept in liquid nitrogen (*22*). Before flow cytometric analysis, samples were stained with Hoechst 33342 (1 μg/ml) according to the method of Monger and Landry (*23*). Samples were analyzed on a Coulter EPICS 541 with the use of 400 mW of UV light. *Prochlorococcus* were discriminated from heterotrophic bacteria and other chlorophyll-containing cells as described previously (*3*).

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