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A Unique Tumor Antigen Produced by a Single Amino Acid Substitution

Paul A. Monach, Stephen C. Meredith, Christopher T. Siegel, and Hans Schreiber
Department of Pathology
The University of Chicago
Chicago, Illinois 60637

Summary

Mice immunized against a cancer recognize antigens unique to that cancer, but the molecular structures of such antigens are unknown. We isolated CD4+ T cell clones recognizing an antigen uniquely expressed on the UV-induced tumor 6132A; some clones inhibited the growth of tumors bearing the specific antigen. A T cell hybridoma was used to purify this antigen from nuclear extracts by RP-HPLC and SDS-PAGE using T cell immunoblot assays. A partial amino acid sequence was nearly identical to a sequence in ribosomal protein L9. The cDNA sequence of L9 from 6132A PRO cells differed from the normal sequence at one nucleotide; this mutation encoded histidine instead of leucine at position 47. A synthetic peptide containing this mutation was over 1000-fold more stimulatory of T cells than was the wild-type peptide. These results indicate that this unique tumor antigen is derived from a single amino acid substitution in a cellular protein.

Introduction

T lymphocytes from many cancer patients display at least rudimentary reactivity against the malignancies of the hosts. Recently, several shared tumor antigens recognized by these T cells or by T cells from mice immunized with cancer cells have been identified at the molecular level (Van der Bruggen et al., 1991; Van den Eynde et al., 1991; Brichard et al., 1993; Gaugler et al., 1994; Bakker et al., 1994; Kawakami et al., 1994; Mandelboim et al., 1994). Each of these antigens has also been found in other tumors or cell lines similar in origin to the tumor from which the antigen was isolated; occasionally, the antigens are expressed by cancers from different lineages as well. In general, the proteins are known to be identical to their counterparts in normal tissues.

The above findings on shared tumor antigens cannot, however, account for the results of experiments that explored acquisition of immunity to tumors: immunization of animals against tumors usually confers specific immunity against that particular tumor and not others, even those of the same tissue type induced with the same carcinogen (Prehn and Main, 1957; Pasternak et al., 1964; Basombrío, 1970; Kripke, 1974; for review see Srivastava and Old, 1988). Such specificity indicates the existence of "unique" or individually distinct tumor transplantation antigens, as opposed to "shared" or tumor-associated transplantation antigens. The diversity of unique tumor antigens is so striking that Burnet (1970) has compared it with that of immune receptors or histocompatibility antigens.

Molecular characterization of unique tumor antigens, in contrast with that of shared antigens, is still at an early stage. It is possible that these unique antigens, which are recognized by T cells of animals immunized against cancer cells (Ward et al., 1989; Cohen et al., 1994), can indeed arise through somatic mutation of cell proteins. Such a protein might become immunogenic after mutation, while the host would likely be tolerant of its normal counterpart. Some of the mutant proteins known to be present in cancer cells, such as mutant Ras and p53, can be used to induce immunity and inhibit tumor growth (Fenton et al., 1993; Noguchi et al., 1994). However, when animals are immunized with tumor cells expressing mutant Ras or p53 proteins, the T cells harvested from these animals do not recognize Ras or p53 (Carbone et al., 1991; Noguchi et al., 1994) but instead recognize other unknown antigens. Thus, mutant Ras and mutant p53 proteins are not the unique tumor antigens responsible for the strong immunity induced by immunization with tumor cells.

Most investigators have used CD8+ cytolytic T lymphocytes (CTL) to define tumor antigens, because these cells are required for the rejection of most experimental cancers by naive mice (Udono et al., 1989; Ward et al., 1990) and can be used to transfer immunity from immune to naive mice (Rosenstein et al., 1984; Chou and Shu, 1987). However, immunity to cancer has also been transferred to new hosts using only CD4+ T cells from immune animals (Greenberg et al., 1981; Fujiwara et al., 1984; Ozawa et al., 1987). In two models, one using a plasmacytoma and one a Friend virus–induced leukemia, immune CD4+ cells appear to have caused rejection of malignant cells even in hosts presumably lacking CD8+ cells (Fujiwara et al., 1984; Greenberg et al., 1985). CD4+ T cell lines and clones reacting with unique and shared tumor antigens in vitro have been isolated (Cohen et al., 1994; Rohrer et al., 1994; Topalian et al., 1994), but the effects of these T cells in vivo and the identities of the recognized unique antigens are unknown. We have begun to identify the molecular nature and genetic origins of unique tumor-specific antigens recognized by T cells of mice immunized with tumor cells. In this study, we have used CD4+ T cell clones isolated from mice immunized against an ultraviolet light (UV)-induced cancer to identify, using a novel approach, the recognized unique antigen as ribosomal protein L9 containing a point mutation.

Results

Isolation of CD4+ T Cell Clones and a Hybridoma Recognizing a Unique Tumor Antigen

The method used to isolate CD4+ clones was adapted from one used to obtain cells recognizing soluble antigens (Gajewski et al., 1989). Mice were immunized with 6132A PRO tumor cells emulsified in complete Freund's adjuvant

Table 1. Response of CD4+T Cell Clones to Tumor Antigens

Clone	Antigen	cpm ± SEM	IL-2	IL-4	IFNγ	TNF/LT
C45-13	None	510 ± 72	360	<3	97000	110
	PRO4L	43622 ± 2182				
	6138	732 ± 75				
	6132A PRO	478 ± 46				
C47-9	None	3635 ± 205	42	<3	22000	110
	PRO4L	3274 ± 325				
	6139B	2601 ± 204				
	6132A PRO	58145 ± 1242				
C47-10	None	3757 ± 885	240	<3	100000	250
	PRO4L	3378 ± 213				
	6139B	3218 ± 224				
	6132A PRO	41131 ± 429				
C47-15B	None	471 ± 83	10	<3	9700	ND
	PRO4L	384 ± 33				
	6138	490 ± 38				
	6132A PRO	11959 ± 1925				
C47-17	None	1104 ± 30	180	<3	81000	90
	TR-PRO	958 ± 6				
	6132A PRO	106274 ± 4300				
C47-21	None	531 ± 46	<0.8	210	<60	12
	TR-PRO	573 ± 8				
	6132A PRO	7502 ± 229				
C417-5	None	2035 ± 81	210	<3	36000	140
	PRO4L	2017 ± 23				
	6139B	2367 ± 523				
	6132A PRO	46653 ± 1863				

Thymidine incorporation assay (columns 2–4). T cell clones were incubated with syngeneic spleen cells and tumor cell extracts at a concentration of 3 × 10^5 cell equivalents per ml. [^{3}H]thymidine was added at about 24 hr, and the cultures were harvested at 40–48 hr. The numbers are the mean counts per minute (*cpm*) ± standard errors (SEM) of triplicate cultures in representative experiments. Cytokine assays (columns 5–8). T cell clones were cultured for 48 hr in wells coated with anti-CD3 MAb 2C11. Supernatants were tested for the presence of IL-2 and IL-4 by stimulation of CTLL-2 cells, of γIFN by ELISA, and of TNF/LT by cytolysis/cytostasis of 1591 RE3.5 cells. The numbers are U per ml, rounded to two significant figures, calculated by comparison to assays with recombinant cytokines in representative experiments. ND, not determined.

(CFA). Cells from the draining lymph nodes were restimulated with tumor cell lysates in vitro and then cloned by limiting dilution (see Experimental Procedures). We obtained clones that synthesized DNA in the presence of lysates of 6132A PRO cells but not lysates of other UV-induced skin tumors that had also originated in C3H mice (Table 1). When stimulated by anti-CD3 antibodies, most of the clones secreted interleukin-2 (IL-2), interferon-γ (IFNγ), and tumor necrosis factor (TNF) or lymphotoxin (LT), and one secreted IL-4 (Table 1). These clones thus appeared to fall into the categories of Th1 and Th2 cells, respectively (Mosmann et al., 1986). By analogous procedures, we isolated a Th1 clone recognizing the 1591 tumor and its progressor variant PRO4L. All clones expressed CD4 by immunofluorescent staining and flow cytometry. Among the 6132A-specific clones, at least three different T cell receptors (TCR) were represented, as 5 of 6 clones were stained with antibodies binding Vβ6 (C47-9, C47-17, C47-21) or Vβ14 (C47-10, C47-15B), whereas C417-5 was not stained with either antibody. Stimulation of clones was found repeatedly using lysates of cells that appeared to be free of mycoplasma by fluorescent staining, indicating that mycoplasma was unlikely to be the source of antigen.

Because a T cell hybridoma would be easier to maintain and prepare for assays than a T cell clone, and therefore would be more useful for assessing the purification of a tumor antigen, we fused the 6132A-specific clone C47-9 with BW5147. Hybridoma 479H-60 secreted IL-2 in response to small amounts of lysates of 6132A PRO; it did not secrete detectable IL-2 in response to lysates of eight other UV-induced tumors, nor to lysates of autologous fibroblasts (Figure 1). These results suggested that the antigen recognized by this hybridoma might be uniquely expressed by 6132A PRO as well as by 6132A, the parental regressor tumor from which the more aggressive variant arose (data not shown). The lack of stimulation by autologous fibroblasts or by 6132B, a second UV-induced skin tumor that arose independently at a different site in the same mouse as did 6132A, suggested that the antigen was not encoded by a germline mutation particular to the mouse of tumor origin.

Determination of Requirements for Processing and Presentation

Conventional antigens recognized by CD4+ cells are peptides of 12–24 aa associated with major histocompatibility complex (MHC) class II molecules (Rudensky et al., 1991; Hunt et al., 1992; Chicz et al., 1992). At least two other types of antigen, however, could be produced by cancer cells. Superantigens are proteins that bind to MHC class

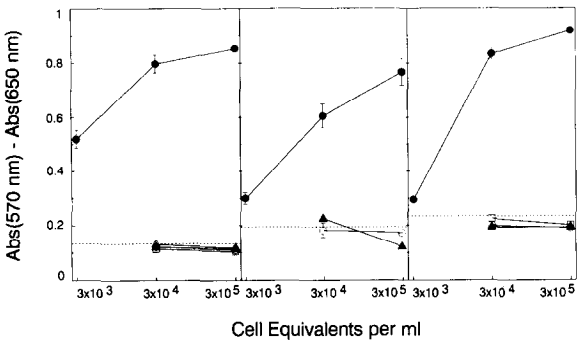

Figure 1. Unique Specificity of a T Cell Hybridoma for an Antigen on 6132A PRO Cells

T cell hybridoma 479H-60 was incubated with C3H spleen cells and lysates of various cells, and IL-2 in supernatants was measured by stimulation of CTLL-2 cells. Left: 6132A PRO (closed circle), 6138 (open circle), 6139B PRO (closed triangle), 4102 PRO (open square), 1591 PRO4L (open triangle). Middle: 6132A PRO (closed circle), 6134A PRO (open circle), 6132 HLF (closed triangle; nontransformed autologous fibroblasts). Right: 6132A PRO (closed circle), 6132B (open circle; autologous second tumor), AG104A (closed triangle), 6130 (open square). Each point represents the mean of duplicate (left) or triplicate (middle and right) wells, with error bars showing the range or SEM, respectively. The means of wells without antigen are shown as dotted lines. Errors less than 0.02 are not shown.

II molecules outside of the peptide-binding groove and to particular TCR Vβ structures (for review see Herman et al., 1991); in this way, they can stimulate a large percentage of the lymphocytes of an animal. Modified MHC class II molecules, associated with a variety of peptides, could similarly stimulate many $CD4^+$ cells in a manner analogous to an allogeneic mixed lymphocyte reaction.

We investigated the MHC class II restriction of 6132A-specific clones in three ways. First, DNA synthesis by all of the 6132A-specific clones shown in Table 1 was completely blocked by monoclonal antibody (MAb) 14-4-4S, which binds $I\text{-}E^k$, but not by MAb 10-2-16, which binds $I\text{-}A^k$ (Figure 2A). Second, the T cell hybridoma 479H-60 recognized antigen presented by cells transfected with $I\text{-}E^k$ but not by cells transfected with $I\text{-}A^k$ (Figure 2B). Third, transfection of cDNAs encoding $I\text{-}E^k$ into 6132A PRO cells sensitized these cells for lysis by clone C47-9 (Figure 2C). All of the 6132A-specific Th1 clones had previously been found to be cytolytic toward $I\text{-}A^{k+}/I\text{-}E^{k+}$ cells incubated with lysates of 6132A PRO, with an efficiency similar to or higher than that shown in Figure 2C. The Th2 clone C47-21 was not cytolytic (data not shown).

Conventional antigens, unlike superantigens, require intracellular processing to be recognized in association with MHC class II. The lysosomotropic drug chloroquine blocks this process (Allen and Unanue, 1984). We found that chloroquine also blocked presentation of 6132A antigen (Figure 3). In addition, 6132A antigen was not presented by BALB/c ($H\text{-}2^d$) spleen cells (data not shown); superantigens usually can bind to multiple MHC class II alleles (DeKruyff et al., 1986; Fleischer and Schrezenmeier, 1988). Together, these results suggested that the 6132A antigen was a processed peptide associated with $I\text{-}E^k$.

Inhibition of Tumor Growth by Th1 Clones

The 6132A PRO tumor grows progressively in only 10%–60% of immunocompetent mice receiving tumor transplants (Ward et al., 1990; data not shown). It was therefore impractical to use $CD4^+$ clones to try to inhibit tumor growth in normal mice, because of the large numbers of mice needed to see significant differences between groups. However, in mice with severe combined immunodeficiency (SCID), a genetic defect that results in the absence of T and B lymphocytes, this tumor always grows progressively, even in mice inoculated with low numbers of cells. One clone recognizing 6132A (C47-17) and one recognizing the 1591 lineage (C45-13) each inhibited, in C3H SCID mice (Figure 4), the growth of the tumor that it recognized in vitro. As measured by immunofluorescent staining and flow cytometry, neither tumor cell line expressed detectable $I\text{-}A^k$ or $I\text{-}E^k$ when cultured with or without IFNγ. Although the 6132A-specific clone appeared to be unable to cause complete rejection, its inhibition of tumor growth in mice lacking $CD8^+$ T cells is remarkable, because 6132A grows progressively in nearly 100% of naive mice depleted of $CD8^+$ cells (Ward et al., 1990). In addition to indicating an anti-tumor potential for Th1 cells, these experiments suggested that the 6132A antigen was released from a growing tumor and gained access to antigen-presenting cells (APCs) in the absence of adjuvant.

Purification of an Antigen from 6132A Cells

Fractionated lysates of 6132A PRO cells were tested for antigenicity by coculturing them with T cell hybridoma 479H-60 and C3H spleen cells as a source of APCs, and then assaying the supernatant for the presence of IL-2. We took advantage of the observation that spleen cells are capable of endocytosing and presenting proteins bound to nitrocellulose (Young and Lamb, 1986). The 6132A antigen was found in the nuclear fraction of disrupted cells and could be solubilized with 2 M but not 0.4 M NaCl (Figure 5A). High salt nuclear extracts were fractionated by reverse-phase high pressure liquid chromatography (RP–HPLC) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE); antigenic activity was eluted with 40%–45% acetonitrile and appeared to reside in a protein of molecular mass 20–22 kDa (Figures 5B and 5C). Based on these findings, we used these techniques in the following sequence to purify the protein for amino acid sequencing: isolation of the nuclear fraction, preextraction with 0.4 M NaCl (discarded), extraction with 2 M NaCl, RP–HPLC, and SDS–PAGE of antigenic HPLC fractions.

Identification of Ribosomal Protein L9 as the 6132A Tumor Antigen

The amino acid sequence of a tryptic fragment of the purified protein was obtained (DFNHINVELSHLGK) and was found to be nearly identical to amino acids 37–50 of ribosomal proteins L9 from both rat and human (DFNHINVELSLLGK; Suzuki et al., 1990; Hori et al., 1993). Indeed, antigenic activity was found at a high level in ribosomes isolated from 6132A PRO cells, and could be purified by the same techniques used to isolate it from the nuclear

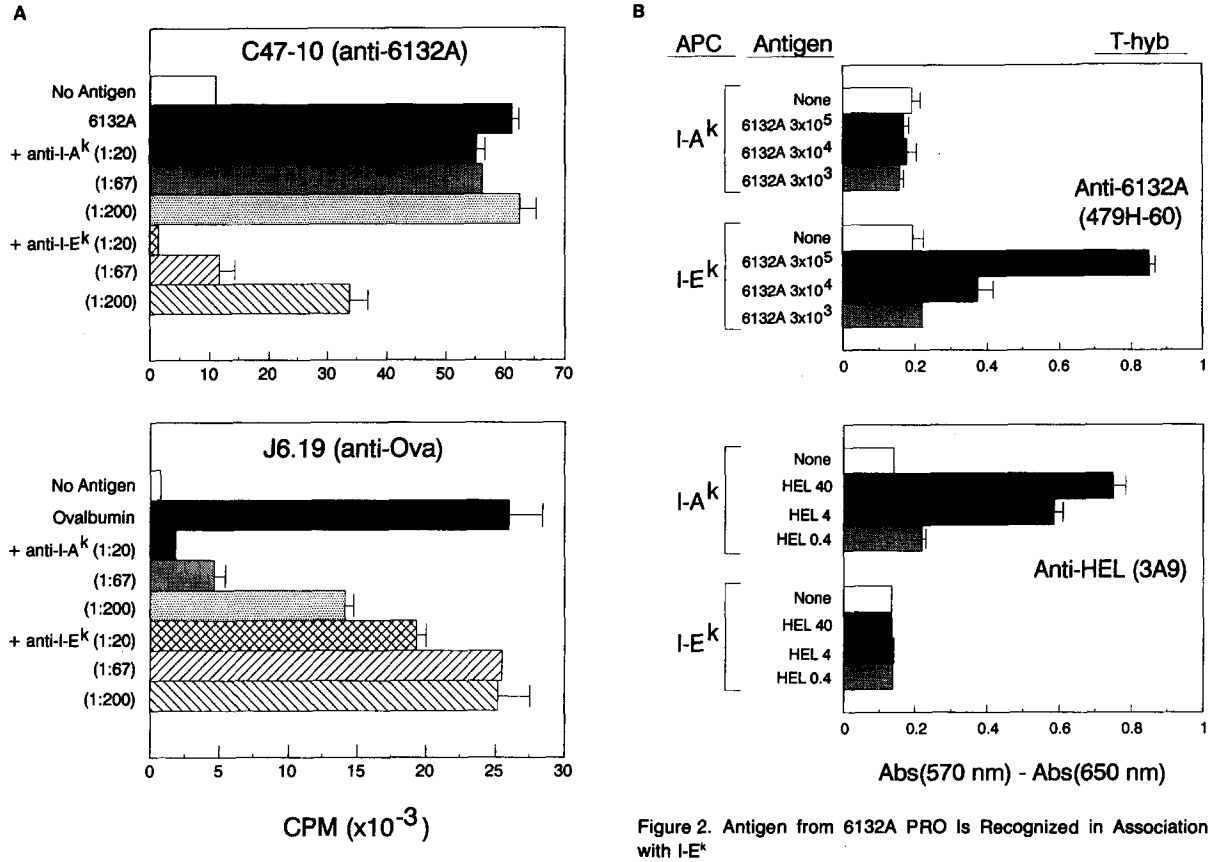

Figure 2. Antigen from 6132A PRO Is Recognized in Association with I-E^k

(A) I-E^k restriction of an anti-6132A PRO T cell clone. Clone C47-10 was incubated with C3H spleen cells and 3 × 10^5 cell equivalents per ml of dead 6132A PRO cells, with or without different amounts of culture supernatants containing MAb 10-2-16 (anti-I-A^k) or 14-4-4S (anti-I-E^k). Clone J6.19, which recognizes ovalbumin in association with I-A^k, was incubated with C3H spleen cells and 800 μg/ml ovalbumin, with or without antibodies as above. The amount of stimulation was measured by thymidine incorporation assay. Error bars show the SEM of triplicate wells; errors less than 1000 cpm (C47-10) or less than 500 cpm (J6.19) are not shown. Thymidine incorporation by all of the other 6132A-specific clones shown in Table 1 was similarly inhibited by anti-I-E^k antibody.

(B) I-E^k restriction of the anti-6132A PRO T cell hybridoma. T cell hybridoma 479H-60 was incubated with different numbers of cell equivalents per ml of dead 6132A PRO cells and with L cells transfected to express either I-A^k (MT58.1.4) or I-E^k (DcEKH1.7) as APCs. T cell hybridoma 3A9, recognizing HEL associated with I-A^k, was incubated with different numbers of micrograms per milliliters of HEL and the same APCs. IL-2 released into supernatants was measured. Error bars show the SEM of triplicate wells; errors less than 0.01 are not shown. Neither hybridoma secreted detectable IL-2 in response to the other antigen (data not shown).

(C) Lysis of I-E^{k+} 6132A PRO cells by a CD4$^+$ anti-6132A T cell clone. Transfectants of 6132A PRO expressing (TR2.34, closed circle; TR2.38, closed square) or not expressing (TR2.8, open circle) I-E^k were tested for sensitivity to lysis by clone C47-9 in a 4.5 hr ^{51}Cr-release assay.

fraction (Figures 5D–5F). Because the nucleotide sequences of the rat and human L9 coding sequences are 91.5% identical (Hori et al., 1993), we reasoned that the mouse sequence should also be very similar to that of the rat. We designed primers for the polymerase chain reaction (PCR) based on the 5′ and 3′ sequences of the coding region of rat L9 and used them to amplify, from cDNA from 6132A PRO, a fragment of about 600 bp, the expected size of the L9 coding sequence plus primers. Using restriction enzyme sites incorporated in the PCR primers, this fragment was cloned into pCA, a bacterial plasmid vector allowing inducible expression of cloned

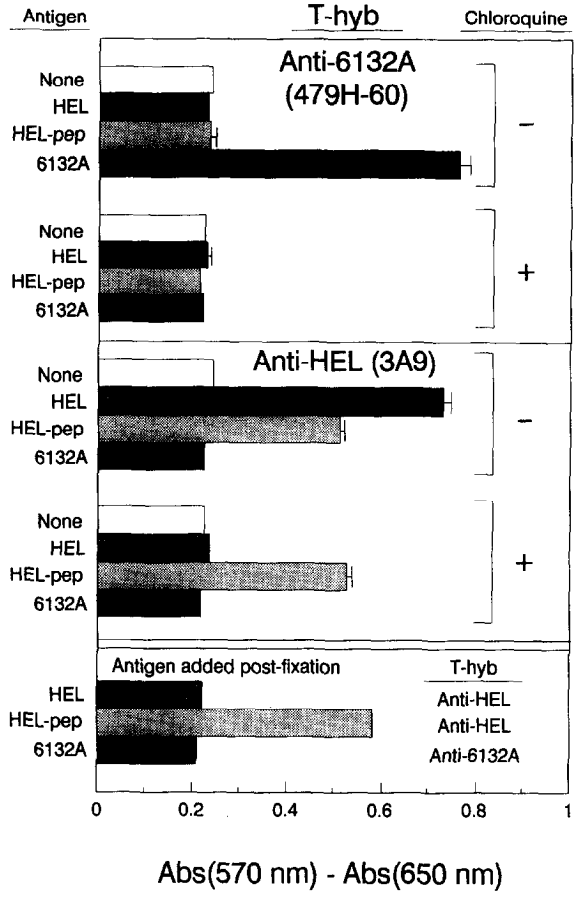

Figure 3. Chloroquine Blocks Presentation of Antigen from 6132A PRO Cells

Spleen cells were treated with chloroquine, pulsed with antigen, and fixed with paraformaldehyde, as described in Experimental Procedures. T cell hybridomas recognizing 6132A (479H-60, top) or HEL (3A9, middle) were added, and 24 hr supernatants were tested for IL-2. Antigens were 2 × 10^6/ml dead 6132A PRO cells, 1 mg/ml HEL, or 0.1 mg/ml HEL digested with trypsin (HEL-pep). The bottom panel shows that cells treated with chloroquine and fixed were still capable of presenting peptide antigen to 3A9, but were incapable of presenting intact HEL or 6132A antigen to the appropriate hybridoma. Error bars show the SEM of triplicate wells; errors less than 0.01 are not shown.

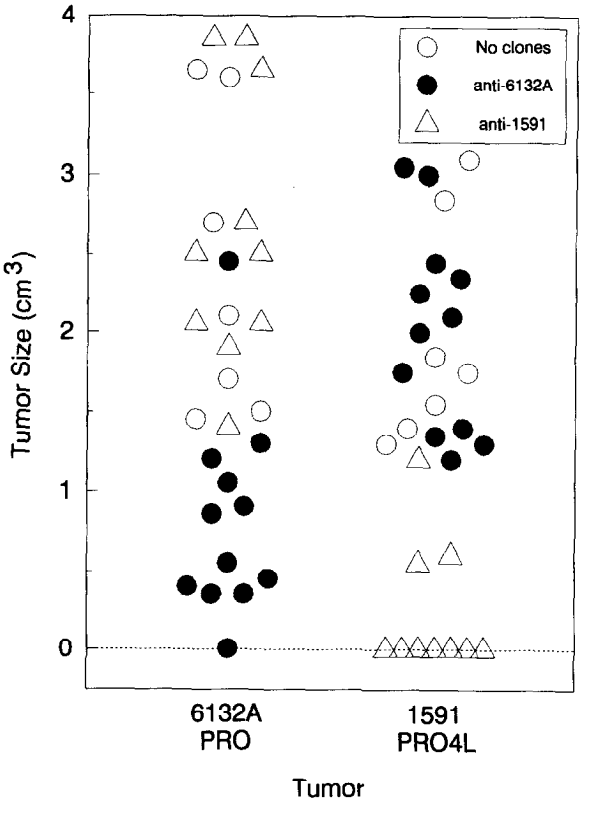

Figure 4. Antigen-Specific Inhibition of Tumor Growth by CD4+ Clones in SCID Mice

C3H SCID mice, unmodified (open circle) or given intraperitoneal injections of 4–5 × 10^7 clones recognizing 6132A (C47-17, closed circle) or 1591 (C45-13, open triangle) received subcutaneous injections of 3 × 10^5 PRO4L cells and either 10^5 6132A PRO or 10^7 6132A RE cells, on opposite flanks. Measurements at 21–24 days after challenge were pooled from three experiments that produced similar results despite slightly different conditions. The first experiment included four mice receiving C47-17, three receiving C45-13, and three untreated, all challenged with 6132A PRO. The second experiment had four mice each with clones and two without, but half of each group received 6132A RE instead of 6132A PRO. The third experiment included four mice with C47-17 and three each untreated or with C45-13; an additional injection of clones was given 7 days after the first. Statistics were calculated for individual experiments using Student's t tests. Sizes of 6132A PRO were significantly smaller in mice treated with C47-17 in experiments one and three ($p < 0.03$; $p < 0.02$), and sizes of PRO4L were significantly smaller in mice treated with C45-13 in all experiments ($p < 0.005$; $p < 0.005$; $p < 0.05$). Each p value is the larger of the two calculated by comparing the experimental group with the two control groups. Results for growth of 6132A RE and PRO in experiment two were similar to those in the other experiments, but differences between these small groups were not significant.

genes as fusion proteins linked to Staphylococcal protein A encoded by the plasmid (Buelow et al., 1992). Lysates of recombinant bacteria stimulated 6132A-specific clones and hybridoma 479H-60 at a high level, but a control lysate was also slightly stimulatory (data not shown). To reduce this background stimulation, fusion proteins were partially purified from lysates by SDS–PAGE. Bands found in the recombinant lysate and not in the control lysate stimulated a 6132A-specific clone but not a 1591-specific clone (Figure 6), strongly suggesting that L9 encoded the 6132A antigen.

Sequencing of L9 cDNA

The 5′ and 3′ rat L9 primers were used to amplify, by reverse transcription (RT)–PCR, L9 coding sequences from RNA from 6132A PRO and from autologous fibroblasts (6132 HLF). Fragments of about 600 bp were cloned into several vectors, and these templates were used for DNA sequencing. We were able to obtain sequences corresponding to nucleotides 38–556 of the rat and human L9 coding regions (Figure 7). The 6132 HLF (wild-type mouse) sequence differed from that of the rat at 29 nt, but only one of these alterations should result in an amino acid change, the conservative shift Asp^{17}→Glu. The 6132A PRO (tumor) sequence differed from the wild-type mouse sequence at a single nucleotide: A instead of T at position 140. The corresponding predicted amino acid sequence contained histidine instead of leucine at position 47; this

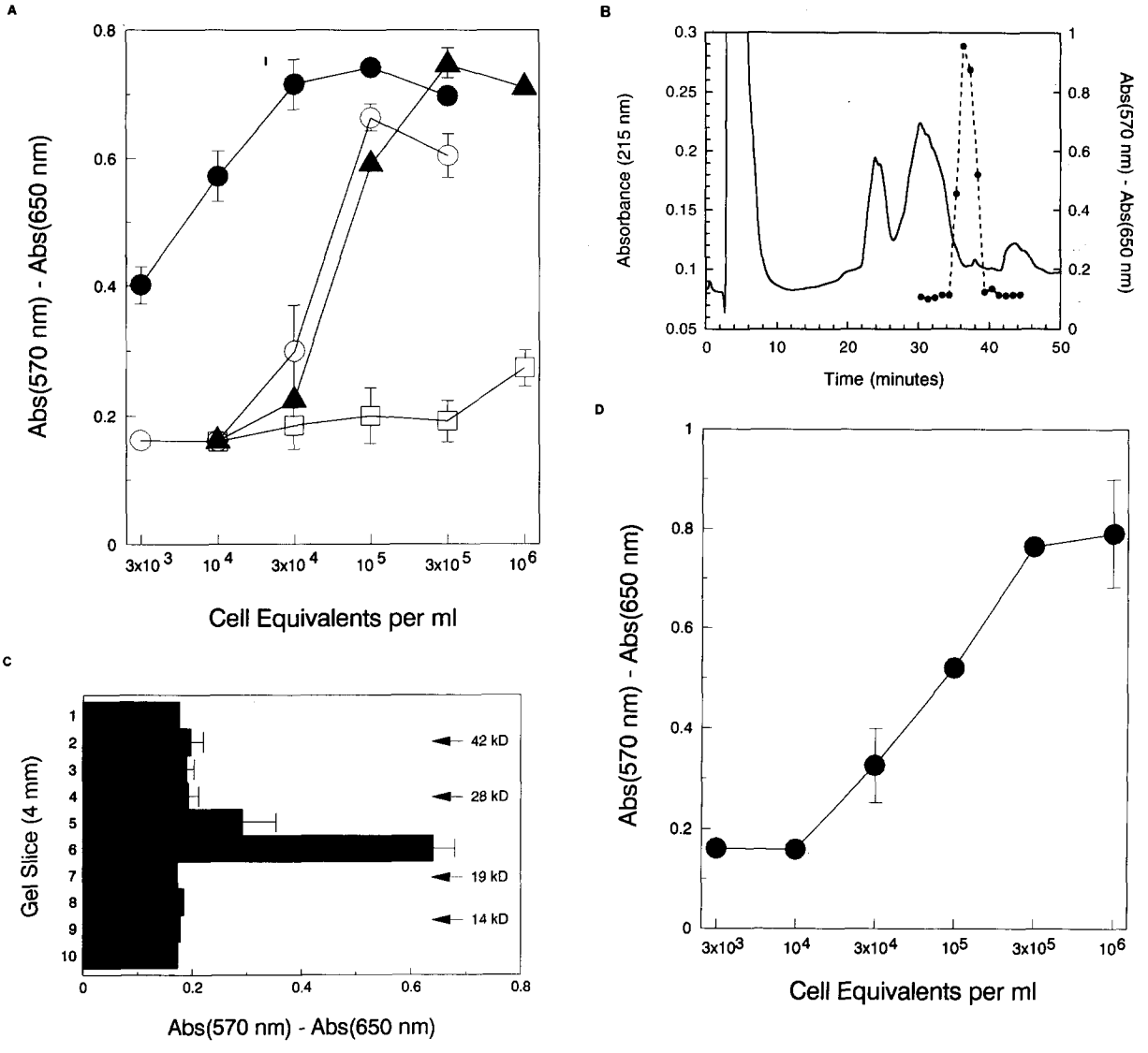

Figure 5. Purification of an Antigen from Nuclei or Ribosomes from 6132A PRO Cells

(A) Hybridoma stimulation assay of subcellular fractions of 6132A PRO cells. Whole cell lysate (closed circle), nuclei (open circle), 0.4 M NaCl nuclear extract (open square), and 2 M NaCl (post-0.4 M NaCl) nuclear extract (closed triangle) were tested for stimulation of IL-2 release from T cell hybridoma 479H-60. Error bars show the range of duplicate wells; errors less than 0.02 are not shown.

(B) Hybridoma stimulation assay of RP-HPLC fractions. A 2 M NaCl nuclear extract of 6132A PRO cells (1.3×10^8 cell equivalents) was injected onto a C4 column in 20% acetonitrile, 0.1% TFA in five aliquots. Material eluting during a gradient of 35%–45% acetonitrile (minutes 10–50; left axis, solid line) was collected. Fractions (1 ml each) at minutes 31–45 were dried and resolubilized in urea–Tris–2-ME, and 10% of each was blotted onto nitrocellulose to test for stimulation of IL-2 release from T cell hybridoma 479H-60 (closed circle, right axis, dotted line).

(C) Hybridoma stimulation assay (T cell immunoblot) of material separated by SDS–PAGE. A 2 M NaCl nuclear extract of 6132A PRO cells (4.8×10^6 cell equivalents) was separated by SDS–PAGE in a Tris–tricine gel system. Proteins were electroblotted onto nitrocellulose, and 4 mm wide strips were cut out and incubated with T cell hybridoma 479H-60. Error bars show the range of duplicate wells; errors less than 0.01 are not shown. Arrows indicate the approximate positions of prestained molecular weight markers.

(D) Crude ribosomes were prepared from 6132A PRO cells as the dense component of the deoxycholate-treated postnuclear supernatant of cells disrupted with NP-40, and were tested for stimulation of IL-2 release from T cell hybridoma 479H-60. Error bars show the range of duplicate wells; errors less than 0.02 are not shown.

(E) Crude ribosomes (2.1×10^8 cell equivalents) were separated by RP–HPLC and fractions collected and assayed as in (B).

(F) The remaining 90% of HPLC fractions 35–45 assayed in (E) were separated by SDS–PAGE in Tris–tricine. Proteins were electroblotted onto nitrocellulose and stained with Ponceau S. A prominent band of the expected size (arrow, right) appears in the antigenic fraction 44 (circled; see [E]); the positions of prestained molecular weight markers (mw) are shown by the arrows at the left. No hybridoma stimulation assay was performed.

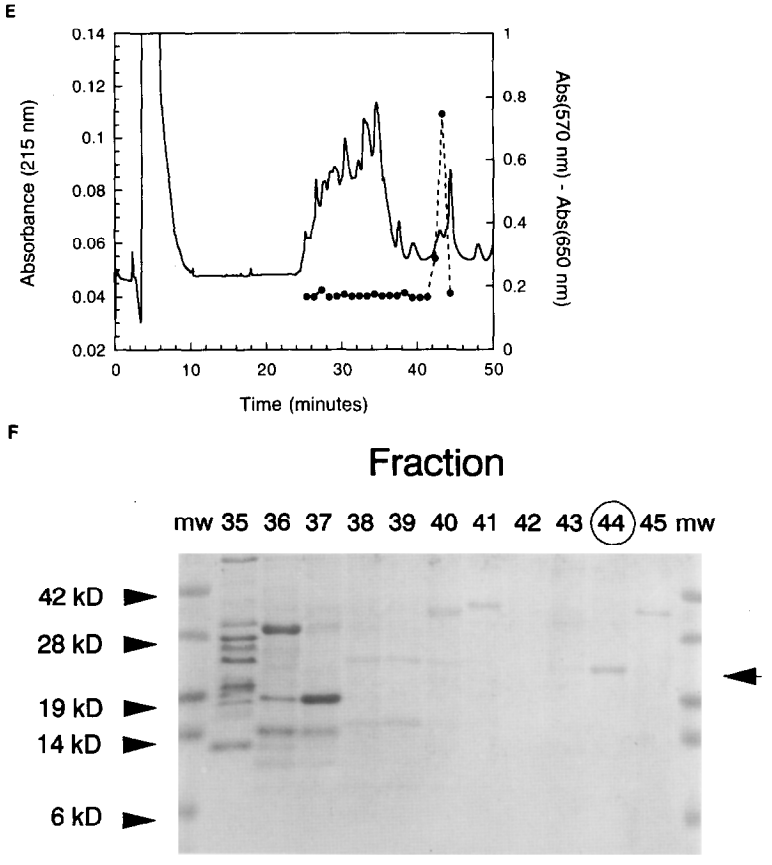

Figure 5

histidine had already been seen by amino acid sequencing of a tryptic peptide (see above). Six HLF L9 cDNA clones from two PCR reactions all featured T at position 140; five 6132A PRO cDNA clones from two PCR reactions all featured A at position 140. In addition, all three cDNA clones sequenced from a different tumor, 6132B, which originated in the same mouse, contained the wild-type T at position 140.

Two other nucleotide differences, one among the HLF and one among the 6132A PRO cDNAs, were found only in individual isolates; thus, these mutations are likely to have occurred during PCR amplification. RT-PCR of RNA from HLF, 6132A PRO, and 6132B also amplified, in addition to the obvious L9 homolog, several closely related sequences (data not shown). We have not investigated these sequences further, and we cannot rule out the possibility that they were amplified from contaminating genomic DNA rather than from mRNA.

Use of Synthetic Peptides to Define the Antigenic Sequence

The results of DNA sequencing suggested that the antigen from 6132A PRO cells was a mutant ribosomal protein L9 containing a histidine residue at position 47. To test this idea, we synthesized peptides of 25 residues centered on position 47 and containing either histidine (6132A-L9p: NH_2-LRRDFNHINVELS*H*LGKKKKRLRVD-$CONH_2$) or leucine (Norm-L9p: NH_2-LRRDFNHINVELS*L*LGKKKKRLRVD-$CONH_2$) at that position. The mutant peptide stimulated release of IL-2 from T cell hybridoma 479H-60 over 1000-fold more effectively than did the normal peptide (Figure 8A). All of the 6132A-specific clones listed in Table 1 were highly activated by 6132A-L9p and less so by Norm-L9p; the 1591-specific clone C45-13 did not increase DNA synthesis in response to either peptide (Figure 8B).

Rejection of a Tumor Challenge by T Cells Induced by Immunization with the Synthetic Mutant Peptide

Immunization of mice with the mutant L9 peptide (6132-L9p) emulsified in CFA induced lymph node cells that proliferated in response to a lysate of 6132A PRO cells but not to a lysate of 1591 PRO4L cells (Figure 9A). Lymph node cells from mice immunized with the same amount of normal L9 peptide (Norm-L9p) did not increase DNA synthesis in response to either tumor cell lysate (data not shown). Cells generated by immunization with the mutant peptide and restimulation in vitro with 6132A PRO lysate caused regression of 6132A PRO in SCID mice but appeared to have no effect on the growth of 1591 PRO4L (Figure 9B). Thus, immunization with the mutant L9 peptide induced lymph node cells capable of antigen-specific tumor rejection.

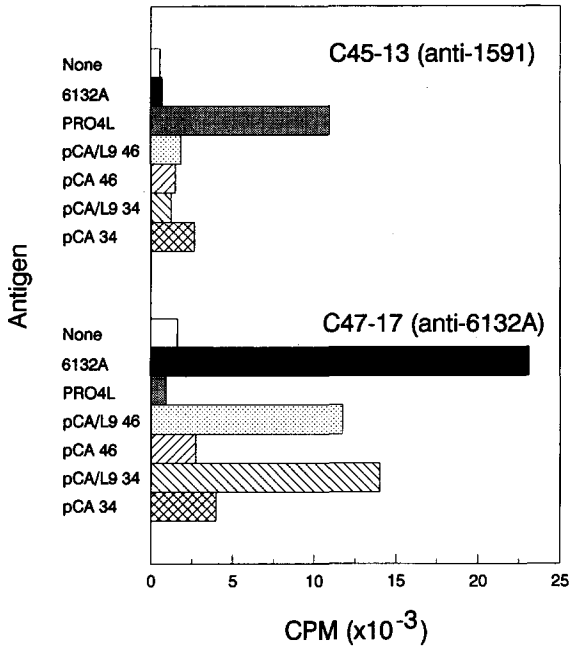

Figure 6. Ribosomal Protein L9 from 6132A PRO Expressed in Bacteria Stimulates T Cells Specific for 6132A

E. coli strain TG1 was transformed with the plasmid vector pCA or with pCA ligated to the L9 coding sequence amplified from 6132A PRO cells by PCR (pCA/L9). Bacterial lysates were solubilized with urea, separated by SDS–PAGE, and the proteins transferred to nitrocellulose. Prominent bands found only in the lysate of recombinant bacteria (pCA/L9 46 and pCA/L9 34) were cut out and incubated with clones specific for 1591 (C45-13) or 6132A (C47-17), as were the corresponding regions from the electrophoresed control lysate (pCA 46 and pCA 34). A band appearing only in the control pCA lysate and of the predicted size of protein A did not stimulate the clones (data not shown).

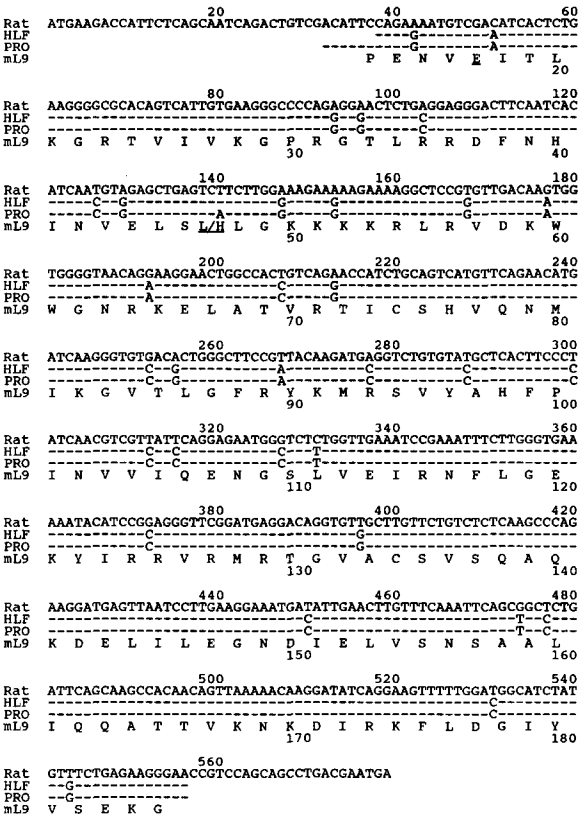

Figure 7. Sequences of L9 cDNAs from 6132A PRO and Autologous Fibroblasts

Top line (Rat): published nucleotide sequence of cDNA encoding rat ribosomal protein L9 (Suzuki et al., 1990). Second line (HLF): consensus sequence of L9 PCR products, amplified from nontransformed autologous fibroblasts using primers corresponding to rat 5′ and 3′ sequences. Third line (PRO): consensus sequence of L9 PCR products amplified from 6132A PRO cells. Fourth line (mL9): deduced partial amino acid sequence of mouse ribosomal protein L9. The only amino acid difference between normal mouse and rat L9 proteins among amino acids 13–185 is at position 17, which is glutamate in mouse (underlined) and aspartate in rat. The only nucleotide difference between normal mouse and tumor cDNA among positions 38–556 is at position 140; this substitution encodes a histidine rather than a leucine at amino acid 47 (underlined).

Discussion

We have isolated CD4+ T cells that recognize a unique antigen made by the UV-induced skin cancer 6132A, and we have used these T cells to identify this antigen at the molecular level. A single amino acid substitution in mouse ribosomal protein L9 appears to be the source of antigenicity, which provides a good explanation for the apparently unique expression of this determinant on 6132A but not other UV-induced murine tumors. The amino acid substitution of histidine for leucine at position 140 is caused by a single nucleotide change, thymidine to adenosine at position 140. Since this difference was the only one found comparing L9 cDNA from 6132A PRO with those from control tissues from the mouse of tumor origin, it is likely to be the result of somatic mutation. This assertion is supported by the finding that the DNA change is within a string of T and C residues; pyrimidine dimers, which can lead to point mutations, are commonly produced in such sequences by UV irradiation (Brash and Haseltine, 1982; Lebkowski et al., 1985). However, we cannot exclude the possibility that this gene exists in the normal mouse genome without sequencing L9 genomic sequences; since there are 20–23 L9-related sequences in the rat (Suzuki et al., 1990), this task is impractical without knowing which gene is transcribed. Although we do not yet know whether the mutant L9 protein is responsible for rejection of 6132A by unimmunized mice, this antigen is likely to be an immunologically important target, since T cells recognizing it inhibit tumor growth in vivo and were derived from hosts immunized against cancer cells; mice immunized in this way show a life-long tumor-specific immunological resistance.

Several observations and lines of reasoning are consistent with the idea that many unique tumor-specific antigens are mutant proteins. First, the physical and chemical carcinogens inducing tumors bearing unique antigens are mutagens (Weisburger and Williams, 1981), and treatment of cancer cells with a point mutagen has produced immunogenic mutant proteins (Lurquin et al., 1989; Sibille et al., 1990). Second, one would expect that random mutation of coding sequences would yield unique antigens. In

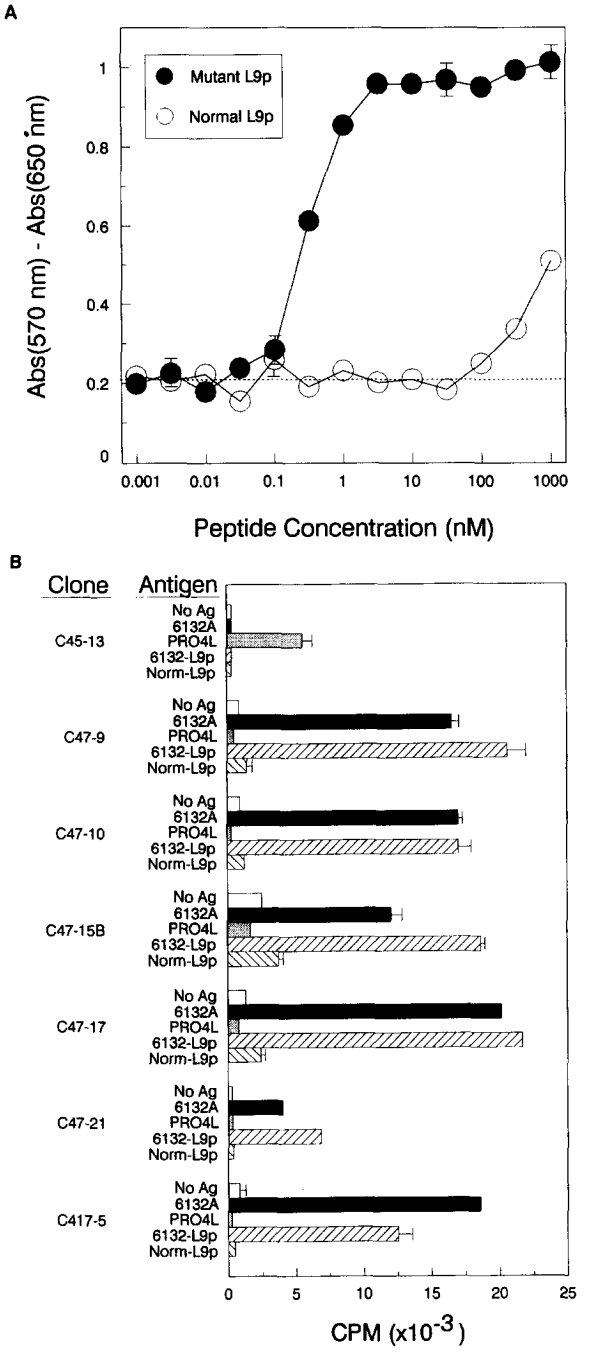

Figure 8. Antigenicity of Peptides Corresponding to Amino Acids 34–58 of L9 Proteins from 6132A PRO and from Normal Cells

(A) Hybridoma stimulation assay. Serial dilutions of 6132A-L9p (closed circle) and Norm-L9p (open circle) were tested for stimulation of IL-2 release from T cell hybridoma 479H-60. The dotted line shows the mean for cultures without peptides. Error bars show the SEM of triplicate samples; errors less than 0.03 are not shown.

(B) Thymidine incorporation assay. T cell clones and spleen cells were incubated without antigen, with 3 x 10^5 cell equivalents per ml of tumor cell lysates (6132A PRO or PRO4L), or with 50 ng/ml of synthetic peptides representing mutant (6132-L9p) or normal (Norm-L9p) L9 sequences. Error bars show the range of two values (No Ag, 6132A, PRO4L) or SEM of three (6132-L9p, Norm-L9p); errors less than 300 cpm are not shown.

contrast, mutations leading to overexpression of otherwise normal proteins would be more likely to produce shared antigens. Third, mutant proteins should, in general, be more immunogenic than normal but overexpressed proteins, because the latter are more likely to be perceived as "self"; consistent with this idea, unique antigens are usually strongly immunogenic. Despite these arguments, however, direct evidence supporting the mutational origin of unique tumor antigens recognized by T cells from hosts immunized against cancer cells has been lacking. This study provides such evidence.

A peptide tumor antigen, associated with MHC class I and recognized by $CD8^+$ T cells, was recently isolated from a spontaneous murine lung cancer cell line (Mandelboim et al., 1994). This antigen was reported to be encoded by a mutant connexin 37 sequence. The T cells reactive toward this peptide appeared to recognize a second spontaneous lung tumor as well, although it is not certain that the antigen on the second tumor is biochemically identical to that on the first. This finding raises the possibility that mutant proteins can act as shared as well as unique antigens.

There are precedents for association of peptides from ribosomal proteins with MHC class I molecules. A nonameric peptide from normal ribosomal protein L28 is one of the major peptides associated with the MHC class I allele HLA-B7 on IG-2 lymphoblastoid cells (Jardetzky et al., 1991), and a nonameric peptide from ribosomal protein L19 is one of the major peptides associated with the nonpolymorphic MHC class Ib molecule SQ7b (Joyce et al., 1994). In addition, exposure of the murine mastocytoma cell line P815 to the point mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in vitro resulted in a mutant ribosomal protein L13a (Sibille et al., 1990; Chan et al., 1994), which was recognized in association with MHC class I by cytolytic T cells. Although the relevance of this antigen to naturally occurring tumor antigens is unknown, the discovery of two independent antigens derived from mutant ribosomal proteins is consistent with the possibility that such proteins, when mutant, commonly become antigenic. One general characteristic of ribosomal proteins is their abundance, and the level of expression of an antigen by cancer cells can affect the induction of immunity (Kündig et al., 1993; Koeppen et al., 1993a). Another common trait of ribosomal proteins is their association with nucleic acids. A variety of nucleic acid–binding proteins, including ribosomal proteins, histones, and components of small nuclear ribonucleoprotein particles, are frequent targets of autoantibodies found in patients with lupus erythematosus and other autoimmune diseases (for review see Naparstek and Plotz, 1993). It is possible that proteins that are highly basic, are complexed with nucleic acids, or are associated with any large macromolecular complex are especially easily taken up by APCs.

Although little is known about the function of ribosomal protein L9, it is possible that mutation of certain ribosomal proteins contributes directly to the initiation or progression of some cancers. For example, a deficiency in the activity of apurinic/apyrimidinic (AP) endonuclease I is associated with one form of xeroderma pigmentosum, in which there

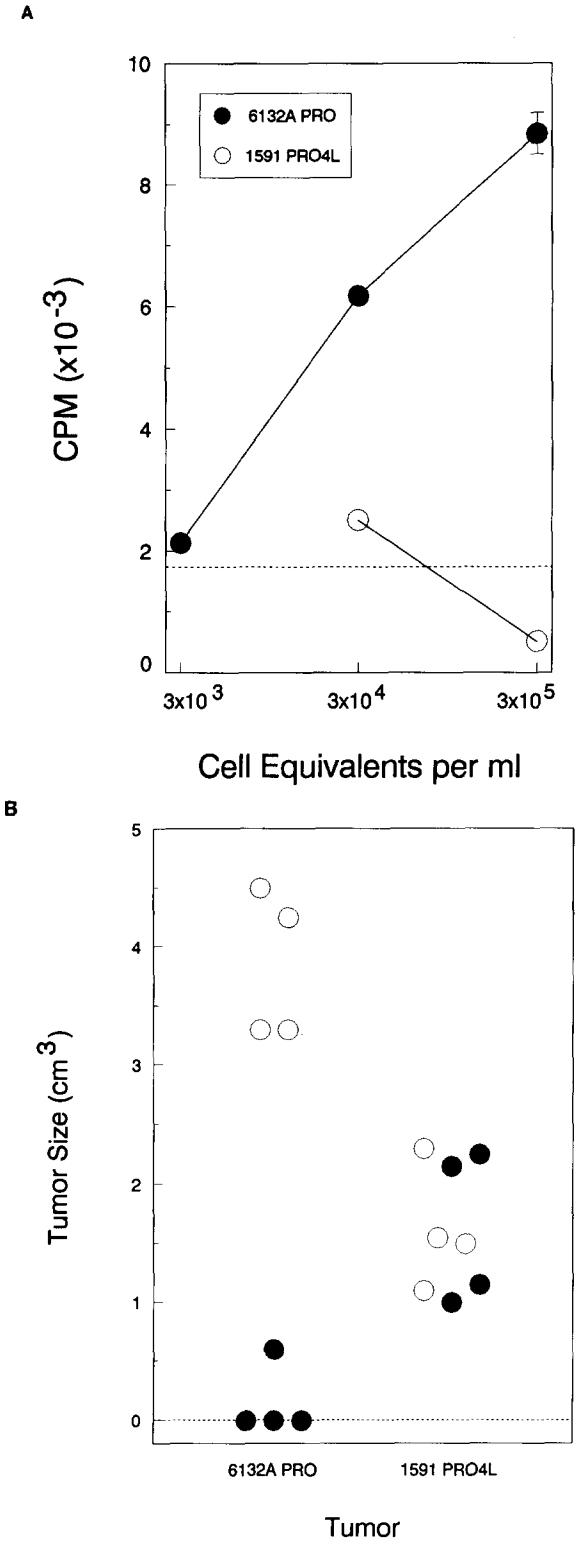

Figure 9. Induction of 6132A-Specific T Cells with a Synthetic Peptide

(A) Mice were immunized with 6132A-L9p in CFA (5 ng per foot) and the draining lymph node cells cultured with different concentrations of lysed 6132A PRO (closed circle) or 1591 PRO4L (open circle) cells. [^{3}H]thymidine was added on day 5 and the cultures harvested on day 6. Error bars show the SEM of triplicate wells; errors less than 200 cpm are not shown. The mean of wells without antigen is shown as a dotted line. Similar results were obtained using mice immunized with 2.5 ng peptide per foot. Lymph node cells from mice immunized with the same amount of normal L9 peptide (Norm-L9p) did not increase DNA synthesis in response to either tumor cell lysate (data not shown).

(B) 6132A-specific cells were generated by immunizing mice with 6132A-L9p (2.5 or 5 ng per foot) and restimulating the draining lymph node cells with lysed 6132A PRO cells. SCID mice, either untreated (open circle) or receiving 8–9 × 10^6 6132A-specific cells intraperitoneally (closed circle), were inoculated with 10^5 6132A PRO cells and 3 × 10^5 1591 PRO4L cells subcutaneously on opposite flanks. Tumor sizes 28 days after challenge are shown, pooled from two experiments, each with two mice per group.

is increased susceptibility to UV-induced skin cancers and other malignancies (Kuhnlein et al., 1976). This enzyme appears to be identical to ribosomal protein S3 (S. Linn et al., submitted). In addition, the *trk-2h* oncogene encodes a fusion of the kinase domain of Trk with part of ribosomal protein L7 (Ziemiecki et al., 1990), and reduced transcription of the gene for ribosomal protein S6 causes overgrowth of the hematopoietic system in Drosophila (Stewart and Denell, 1993). Our results have confirmed that ribosomal protein L9 is highly conserved in evolution (Suzuki et al., 1990; Hori et al., 1993), and it may be necessary for cell survival and high fidelity protein biosynthesis in normal cells. Furthermore, the mutant L9 may also be required for the survival, and therefore the malignancy, of 6132A PRO cells, since we did not find any transcripts for the normal counterpart, nor have we found tumor variants that have lost expression of this mutant protein. The success of immunotherapy may depend on the existence of antigens that cannot be easily lost by cancer cells.

The approach we have developed may be useful for identifying other unique antigens, which are frequently present on tumors caused by chemical or physical carcinogens. Purification of intact proteins should, in general, be easier than purification of peptides eluted from MHC molecules, because of the smaller numbers of cells required and the possibility of fractionating material on the basis of size. Intact proteins might also be assayed for stimulation of tumor-specific CD8$^+$ T cells by hydrolyzing the proteins briefly with alkali (Gavin et al., 1993). It is presently unclear whether our approach of protein purification and T cell immunoblotting, or elution of peptides from MHC molecules (Cox et al., 1994; Mandelboim et al., 1994), or expression cloning in eukaryotic cells (Van der Bruggen et al., 1991) or bacteria (Hickling et al., 1992) will be most useful for identifying tumor antigens. In any case, our method of using CD4$^+$ tumor-specific T cell clones or hybridomas for identifying the genetic origins of tumor antigens may uncover novel mutant proteins in cancer cells that not only are important immunological target molecules, but also are important in the development and progression of malignancy.

Experimental Procedures

Mice and Cell Lines

Female C3H/HeN MTV$^-$ and BALB/c mice were purchased from the Frederick Cancer Research Facility. Male and female C3H SCID mice were provided by J. Bluestone; they were bred at the University of Chicago from mice purchased from the Jackson Laboratory. The C3H

tumors used were the spontaneous fibrosarcoma AG104A (Ward et al., 1989) and UV-induced skin tumors described previously: 6130, 6132A, 6132B, 6138, and 6139B (Ward et al., 1989); 4102 PRO, 6132A PRO, 6134A PRO, and 6139B PRO (Ward et al., 1990); 1591 PRO4L (Urban et al., 1982), 1591 TR-PRO (PRO4L transfected with an allogeneic MHC class I molecule; Koeppen, et al., 1993b), and 1591 RE3.5 (Teng et al., 1991). These cell lines were maintained in MEM with 5%–10% fetal bovine serum (FBS), as were fibroblasts from the heart and lungs of the mouse in which 6132A and 6132B originated (6132 HLF). The 6132A tumor is part of a large tumor library (Ward et al., 1989), which is unique in that we have isolated autologous control cells from each mouse in which a tumor originated. Using these control cells, we can distinguish between somatic and germline mutations. Without such controls, some antigens found to be tumor specific (Philipps et al., 1985) may later be identified as products of polymorphic genes (Lee et al., 1988). Antigen-presenting L cell transfectants MT58.1.4 (Ltk^- cells transfected with $I\text{-}A^k$ and genomic Ii; Peterson and Miller, 1992) and DcEKH1.7 (DAP3 cells transfected with $I\text{-}E^k$ and expressing endogenous genomic Ii) were gifts from J. Miller. T cell hybridoma 3A9 (Allen and Unanue, 1984), recognizing hen egg lysozyme (HEL), and solutions of intact and trypsin-digested HEL were also gifts from J. Miller. $CD4^+$ T cell clone J6.19, recognizing ovalbumin associated with $I\text{-}A^k$ (Wilde and Fitch, 1984), was a gift from F. Fitch, as was CTLL-2, responsive to IL-2 and IL-4 (Gillis and Smith, 1977). BW5147 (Goldsby et al., 1977) was a gift from J. Urban. 3A9, MT58.1.4, and DcEKH1.7 were maintained in DMEM supplemented with 10% FBS (Sigma or GIBCO), 2 mM glutamine (GIBCO), 0.1 mM nonessential amino acids (GIBCO), 10 mM HEPES (GIBCO) or 50 mM MOPS, 5×10^{-5} M 2-mercaptoethanol (2-ME), and penicillin and streptomycin (GIBCO); CTLL-2 was grown in the same medium plus 20–30 U per ml of recombinant human IL-2 (Cetus). BW5147 was grown in RPMI supplemented with 10% FBS, glutamine, nonessential amino acids, HEPES, 2-ME, penicillin and streptomycin, and 1 mM sodium pyruvate (GIBCO). 6132A PRO and 1591 TR-PRO cells were tested for contamination with mycoplasma by staining with Hoechst 33258, followed by fluorescence microscopy.

Generation, Maintenance, and Analysis of $CD4^+$ T Cells

6132A PRO or 1591 TR-PRO cells were washed with MEM, resuspended at a density of $6–13 \times 10^7$ cells per ml in MEM, lysed by three cycles of freezing and thawing, and emulsified with an equal volume of CFA (Difco). About 0.05 ml of emulsion was injected into each hind foot of C3H mice. The popliteal lymph nodes were removed 7 or 8 days later and the cells dispersed and cultured as follows: 2×10^6 lymph node cells, $4–6 \times 10^6$ irradiated (20 Gy) syngeneic spleen cells, and antigen (see below) in a well of a 24-well plate in 1.5 ml of DMEM supplemented with 0.4% fresh syngeneic mouse serum, glutamine, HEPES or MOPS, 2-ME, and penicillin and streptomycin at the same concentrations as above; sometimes, nonessential amino acids, nystatin (GIBCO), and gentamycin (GIBCO) were added at the same concentrations as above. Antigens were different amounts of tumor cell lysates, which were routinely made at concentrations of 6×10^7 cells per ml and were stored at 4°C. After 7–11 days of culture, T cells were cloned by limiting dilution in 96-well plates in the medium above supplemented with 13 U/ml of recombinant human IL-2. After cloning, cells were expanded and passaged in the same medium, but with 10% FBS instead of 0.4% mouse serum; the usual amount of antigen added was 2×10^5 cell equivalents per ml. Clone C45-13 was sometimes grown in the presence of 400 U/ml of recombinant murine IFNγ (Genentech), but its performance was similar with or without this added cytokine.

Expression of cell surface markers was assessed by flow cytometry (FACScan, Becton-Dickinson). The antibodies used were anti-L3T4(CD4) conjugated to phycoerythrin (Becton-Dickinson), anti-Vβ6 conjugated to fluorescein isothiocyanate (FITC; PharMingen), and anti-Vβ14-FITC (PharMingen).

The specificity for antigen of T cell clones was assessed by thymidine incorporation assay. T cell clones were washed twice 9 or more days after the last restimulation with antigen to remove residual IL-2, then cultured as follows: 10^5 T cells and about 10^6 irradiated (20 Gy) syngeneic spleen cells, with or without a tumor cell lysate, in a well of a flat-bottomed 96-well tissue culture plate in 0.2 ml DMEM supplemented as above but without IL-2. About 24 hr later, 1 μCi of [^{3}H]thymidine (Amersham) was added to each well, and about 24 hr after that, the cultures were harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology). Radioactivity was measured by liquid scintillation counting. For some experiments, culture supernatants containing MAb 10-2-16 (anti-$I\text{-}A^k$; Oi et al., 1978) or 14-4-4S (anti-$I\text{-}E^k$; Ozato et al., 1980) were added at the beginning of culture. For other experiments, antigen was added in the form of protein electroblotted onto nitrocellulose, which was removed just before adding [^{3}H]thymidine.

Supernatants for measurement of cytokines were obtained by incubating washed T cells at a concentration of 10^6 cells per ml in plates (24-well or 96-well) coated with anti-CD3 MAb 2C11 (Leo et al., 1987), a gift from J. Bluestone. The 2C11 antibody was applied to wells either as culture supernatant or as a 10 μg/ml solution of affinity-purified antibody. Units of IL-2 and IL-4 were measured by adding dilutions of T cell supernatants to CTLL-2 cells in the presence of MAb blocking either IL-4 (11B11, a gift from F. Fitch; Ohara and Paul, 1985), or IL-2 (S4B6, a gift from R. Coffman; Mosmann et al., 1986) and its receptor (3C7, a gift from F. Fitch; Ortega et al., 1984). IFNγ was measured by enzyme-linked immunosorbent assay (ELISA) (Schreiber et al., 1985), the reagents for which were provided by F. Fitch. TNF/LT was measured by cytolysis/cytostasis of the TNF-sensitive cell line 1591 RE3.5 (Teng et al., 1991).

Cytolysis was measured by 4–5 hr ^{51}Cr-release assay (Urban et al., 1982; Ward et al., 1989). The target cells had been transfected with DNAs encoding I-Eα and $I\text{-}E\beta^k$ (pcEXV-Eα and pcEXV-$E\beta^k$; Miller and Germain, 1986) and neomycin resistance (pSV2-neo). These plasmids were provided by J. Katz, A. Sant, and J. Miller. G418-resistant cells expressing $I\text{-}E^k$ (binding to 14-4-4S) were enriched by magnetic sorting using 14-4-4S and Dynabeads (Dynal) and cloned by limiting dilution. Clones were analyzed for cell surface expression of $I\text{-}E^k$ by flow cytometry, using 14-4-4S and goat anti-mouse IgG–FITC (HyClone). 6132A PRO and 1591 PRO4L cells were also tested for expression of $I\text{-}E^k$ in this way, as well as for expression of $I\text{-}A^k$ by staining with 10-2-16 and goat anti-mouse IgG–FITC.

Generating $CD4^+$ T cells by the methods above requires caution. Immunizing and restimulating with cells cultured in FBS seems to stimulate $CD4^+$ cells recognizing FBS proteins, so that under these conditions, tumor-specific T cells may be found only when the immunizing tumor has a strong antigen. Other investigators have reported similar domination of cultures by cells recognizing FBS proteins or bacterial collagenase used to digest tumors (Cohen et al., 1994). In addition, cell extracts contaminated with mycoplasma greatly stimulate activation of lymph node cells during their initial passage, whether by mitogenic activity or by cross-reactivity with antigens in CFA. Both problems can be avoided by using tumors excised from SCID or nude mice as the sources of antigen (P. A. M., unpublished data).

Generation and Analysis of T Cell Hybridomas

T cell clone C47-9 was fused, 3 days after stimulation with antigen and IL-2, to BW5147 using polyethylene glycol (Goldsby et al., 1977). Isolates resistant to hypoxanthine–aminopterin–thymidine (GIBCO) were tested for secretion of IL-2 in the presence of syngeneic spleen cells and lysates of 6132A PRO cells. Hybridoma 479H-60, which was used later to assess purification of an antigen from 6132A PRO lysates, was maintained in supplemented RPMI. The standard assay for IL-2 secretion by this cell line or by T cell hybridoma 3A9 was performed by culturing about 10^6 irradiated (20 Gy) syngeneic spleen cells in 0.1 ml supplemented DMEM with 0.1 ml of confluent hybridoma culture in a well of a 96-well plate. Antigen was added in the form of tumor cell lysate, fractionated extract, protein blotted onto nitrocellulose electrophoretically or by capillary action (Bio-Dot apparatus; Bio-Rad), pure protein, or synthetic peptide. After about 24 hr of culture, 0.1 ml of culture supernatant was removed and added to washed CTLL-2 cells; about 24 hr later, 0.1 ml of supernatant was removed and discarded, and 0.02 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml) was added to each culture. The following day, 0.1 ml of 10% SDS, 0.01 M HCl was added, and the day after that the absorbance at 570 nm minus the absorbance at 650 nm was measured using a V_{max} kinetic microplate reader (Molecular Devices). This MTT assay (Mosmann, 1983) was used for quantitating bioassays for IL-2, IL-4, and TNF/LT as well (see above). For some assays, MT58.1.4 and DcEKH1.7 were used instead of syngeneic spleen cells as APCs.

For assays involving chloroquine (Allen and Unanue, 1984), spleen cells were first treated for 15 min at room temperature with 0.1 mM chloroquine or with buffer. After antigens were added, the cells were incubated at 37°C for at least 2 hr, then washed, fixed with 1% paraformaldehyde in Hank's balanced salt solution, washed, incubated at 37°C for 1 hr to release residual fixative, and washed again before incubating with T cell hybridomas.

Protein Purification

Nuclei were prepared from cultured tumor cells by disrupting the cells twice with 1% Triton X-100 in RSBI (10 mM Tris–HCl, 10 mM NaCl, 3 mM $MgCl_2$, 300 μg/ml leupeptin, 300 μg/ml aprotinin, 40 μg/ml phenylmethylsulfonyl fluoride [pH 7.5]) for 1 hr at 4°C, and recovering the nuclei by centrifugation at 2000 × g. Nuclei were incubated twice with RSBI plus 0.4 M NaCl for 1 hr at 4°C, then once with RSBI plus 2 M NaCl overnight at 4°C. This last extraction was centrifuged at 200,000 × g for 2–3 hr and the supernatant retained. Ribosomes were prepared as described by others (Sallustio and Stanley, 1990). In brief, the supernatant of cells disrupted with Nonidet P-40 (NP-40) was treated with deoxycholate, then layered on a discontinuous sucrose gradient and centrifuged at 100,000 × g for 24 hr; the pellet, enriched in ribosomes, was saved and resuspended in 20 mM Tris–HCl (pH 7.6), 5 mM magnesium acetate, 100 mM NH_4Cl, 1 mM dithiothreitol, 0.2 M sucrose. Bacterial lysates prepared by digestion and disruption with lysozyme, deoxycholate, and DNAase (Sambrook et al., 1989) were centrifuged at 10,000 × g for 10 min, and the pellets solubilized with 6 M urea at 4°C overnight.

To prepare a nuclear extract or ribosomal fraction for either RP–HPLC or SDS–PAGE, it was mixed with an equal volume of UT2 (6 M urea, 10 mM Tris–HCl [pH 8.5], 2% 2-ME) and an equal volume of 3× SDS (12% (w/v) SDS, 150 mM Tris–HCl [pH 6.8], 15% (v/v) glycerol, 0.03% (w/v) bromophenol blue), with additional 2-ME added to a final concentration of 5% (v/v); this solution was then incubated at 37°C for 1 hr. This unorthodox combination of denaturants was used because it was the only solution we tried that seemed to solubilize the antigenic activity consistently. In particular, we found that high salt nuclear extracts frequently precipitated in the 0.1% trifluoroacetic acid (TFA) used for chromatography. However, the sample that was eventually used to obtain amino acid sequence was not combined with UT2, 3× SDS, or 2-ME before chromatography.

RP–HPLC was performed on an IBM LC/9533 ternary gradient liquid chromatograph with a 4.6 mm × 25 cm C4 column (Vydac). All HPLC experiments were performed using water and acetonitrile (AcCN), both acidified with 0.1% (v/v) TFA, at a flow rate of 1 ml per minute; fractions of 1 min duration were collected. Routinely, a sample was loaded at 20% AcCN in several aliquots of 1 ml each; after the final loading, the concentration of AcCN was increased to 35% using a linear gradient over the first 10 min, then to 45% over the next 40 min; the sample that was used to obtain amino acid sequence was loaded at 35% AcCN and eluted, as above, with a 40 min linear gradient to 45% AcCN. Fractions were dried in a Speed Vac concentrator (Savant), resuspended in 40–80 μl of UT2, and placed at 4°C overnight before using.

Antigen from 6132A PRO cells, whether from nuclei or HPLC fractions, was separated by SDS–PAGE using a Tris–tricine buffer system (Schägger and von Jagow, 1987). The resolving gel was 16.5% T, 3% C, and a spacer gel with 10% T, 3% C was usually included between the resolving and stacking gels. In addition, 10 mM dithiothreitol was included in the resolving and spacer gels, and 0.2% 2-ME was added to the cathode and anode buffers. For separating proteins from bacterial extracts, SDS–PAGE conditions according to Laemmli (1970) were used, with 12% acrylamide in the resolving gel. In either case, an HPLC fraction in UT2 or a bacterial lysate in 6 M urea was prepared for SDS–PAGE by adding one half volume 3× SDS and 2-ME to 5% and incubating at 37°C for 1 hr. Separated proteins were transferred to nitrocellulose at 50–60 mA for 18–24 hr in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol. For obtaining amino acid sequence, proteins were transferred to PVDF (Pro-Blott; Applied Biosystems) under the same conditions. Nitrocellulose and PVDF membranes were stained with Ponceau S.

Protein Sequencing

All steps were performed at the Harvard Microchemistry Facility. Protein bound to PVDF was cleaved with trypsin and the fragments eluted and separated by RP–HPLC. A prominent peak was subjected to Edman degradation, yielding the sequence DFNHINVELSHLGK.

PCR, DNA Cloning, and DNA Sequencing

Oligonucleotides were purchased (Operon) that corresponded to the 5′ sense and 3′ antisense sequences of the coding region of the cDNA for rat ribosomal protein L9, with a BamHI site attached to the 5′ end of the 5′ sense sequence and XhoI and EcoRI sites attached to the 5′ end of the 3′ antisense sequence; the resulting oligonucleotides were 5′-GTCGGATCCATGAAGACCATTCTCAGCAATCA and 5′-CTGAATTCTCGAGGTCTCATTCGTCAGGCTG, respectively. These primers were used to reverse-transcribe RNA from cultured cells and amplify it by PCR. RNA was isolated from 6132A PRO, 6132 HLF, and 6132B cells by the Trireagent method (Molecular Research Center). RNA was mixed with the 5′ and 3′ primers and MMLV reverse transcriptase (GIBCO BRL), and incubated at 38°C for 10 min; after incubating at 94°C for 5 min, Taq polymerase (Promega) was added, and 40 cycles of PCR performed as follows: 1 min at 94°C; 2 min at 55°C; 3 min at 72°C. Products were blunt-ended by an additional 10 min incubation at 72°C at the end of the PCR. All incubations were performed in a Perkin-Elmer Cetus 480 DNA thermal cycler. Double-stranded cDNA was also made from RNA from 6132A PRO cells; RNA isolated with RNAzol (Cinna/Biotecz) was reverse-transcribed with M-MLV-RT using an oligo-dT primer, and the second DNA strand was synthesized with the Klenow fragment of Escherichia coli DNA polymerase I (New England Biolabs). PCR products were either digested with BamHI and XhoI and ligated into pCA (Buelow et al., 1992) or digested with BamHI and EcoRI and ligated into pBluescript II KS (Stratagene) or pCDNA3 (Invitrogen). Recombinant pCA plasmids were electroporated (GenePulser; Bio-Rad) into E. coli strain TG1, and recombinant pBluescript and pCDNA3 plasmids were electroporated into E. coli strain TOP10F′; bacteria containing plasmids were maintained in 2× YT medium with 100 μg/ml carbenicillin.

Plasmid DNA was prepared by standard small-scale techniques (Sambrook et al., 1989) with the inclusion of extractions with phenol, phenol/chloroform/isoamyl alcohol (25/24/1 v/v/v), and chloroform/isoamyl alcohol (24/1 v/v). DNA sequencing by the dideoxy chain termination method (Sanger et al., 1977) was performed with a Sequenase Version 2.0 kit (United States Biochemical) according to the instructions of the manufacturer, using alkaline denaturation of plasmid DNA, either of the two L9 primers described above, and ^{35}S-labeled dATP (Amersham). Two persistent compression artifacts at nucleotide positions 95–96 and 212–215 were resolved by sequencing with dITP using the same procedure.

Synthetic Peptides

The following peptides were synthesized on an Applied Biosystems 431A peptide synthesizer using t-Boc chemistry: NH_2-LRRDFNHINVELS*H*LGKKKKRLRVD-$CONH_2$ (6132-L9p) and NH_2-LRRDFNHINVELS*L*LGKKKKRLRVD-$CONH_2$ (Norm-L9p). Synthetic yields were calculated by quantitative ninhydrin tests of resin samples taken during synthesis; all steps showed >99% yield. Peptides were cleaved from the resin using anhydrous HF/*p*-cresol/resin in a 10/1/1 (v/v/w) mixture. After cleavage, peptides were extracted into 0.5% (w/v) ammonium bicarbonate and desalted by gel filtration chromatography using Sephadex G-25 Superfine in a 95 × 2.6 cm column. Peptides were purified by RP–HPLC using the system, C4 column, and solvents described above. Samples in 0.1% TFA were loaded at 18% AcCN and eluted with a 90 min linear gradient to 28% AcCN; the Norm-L9p peptide eluted at 27–47 min in different runs, and the 6132-L9p peptide eluted at 14–24 min in different runs. The concentrations and compositions of HPLC-purified peptides were determined by amino acid analysis (Heinrikson and Meredith, 1984). Peptides were analyzed by fast atom bombardment mass spectrometry (University of Illinois at Chicago MAT 90 Mass Spectrometry Facility) to verify their identities.

Tumor Challenge

T cell clones were harvested 9 days after the last stimulation with antigen and IL-2, washed once with MEM, and injected intraperitoneally into C3H SCID mice, 4–5 × 10^7 cells per mouse. Immediately thereafter, each mouse received subcutaneous inoculations of 3 × 10^5 PRO4L cells and either 10^5 6132A PRO or 10^7 6132A RE cells, on

opposite flanks. In one experiment, mice received a second injection of clones 7 days after the first. Tumors were measured twice per week starting on day 14 after challenge. The tumor sizes in cubic centimeters were calculated by dividing the product of the three maximum orthogonal diameters by two; this calculation approximates the formula for an ellipsoid,

$$\frac{\pi abc}{6}$$

where a, b, and c are the three maximum orthogonal diameters.

Short-term T cell lines were generated by immunizing mice with synthetic peptides emulsified in CFA (2.5 or 5 ng per foot) and culturing the lymph node cells with tumor cell lysates using the conditions described above for primary cultures. Cells used for thymidine incorporation assays were transferred to 96-well plates and received [^{3}H]thymidine on day 5; cultures were harvested on day 6. Cells for use in vivo were harvested on day 9, washed once with MEM, and injected intraperitoneally into SCID mice (8–9 × 10^6 per mouse). SCID mice subsequently received 10^5 6132A PRO cells and 3 × 10^5 PRO4L cells subcutaneously on opposite flanks. Tumors were measured and sizes calculated as above.

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