Utilization of an Alternative Open Reading Frame of a Normal Gene in Generating a Novel Human Cancer Antigen

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Summary

Tumor infiltrating lymphocytes (TILs) derived from tumor-bearing patients recognize tumor-associated antigens presented by major histocompatibility complex (MHC) class I molecules. The infusion of TIL586 along with interleukin (IL) 2 into an autologous patient with metastatic melanoma resulted in the objective regression of tumor. A gene encoding a tumor antigen recognized by TIL586 was recently isolated and shown to encode gp75. Here we report that an antigenic peptide, MSLQRQFLR, recognized by TIL586 was not derived from the normal gp75 protein. Instead, this nonamer peptide resulted from translation of an alternative open reading frame of the same gene. Thus, the *gp75* gene encodes two completely different polypeptides, gp75 as an antigen recognized by immunoglobulin G antibodies in sera from a patient with cancer, and a 24–amino acid product as a tumor rejection antigen recognized by T cells. This represents the first demonstration that a human tumor rejection antigen can be generated from a normal cellular gene using an open reading frame other than that used to encode the normal protein. These findings revealed a novel mechanism for generating tumor antigens, which may be useful as vaccines to induce tumor-specific cell-mediated immunity against cancer.

T he adoptive transfer of tumor infiltrating lymphocytes (TILs)[1] can mediate tumor regression in patients with metastatic melanoma (1, 2), suggesting that tumor rejection antigens recognized by T cells exist on these tumor cells. The availability of such T cells has made it possible to clone and sequence the genes that encode human melanoma antigens (3–7). The antigens identified so far from human melanoma can be divided into two classes based on their expression pattern. The antigens of the first class are encoded by genes that are expressed only in tumor and testis, but not other normal human tissues. MAGE1, MAGE3, and BAGE are examples of this class (8–10). The second class of antigens represents differentiation antigens encoded by genes that are expressed only in melanocytes, melanomas, and retina. MART-1/Melan-A, gp100, and tyrosinase are examples of this class (11–16). All these antigens are nonmutated self-proteins. Identification of the antigenic epitopes recognized by T cells derived from the corresponding gene products is important not only for understanding the mechanism of immune response to self-antigens, but also for developing new, effective immunotherapeutic strategies with these an-

tigens or synthetic peptides for the treatment of patients with cancer.

Previous studies showed that the infusion of TIL586 plus IL-2 into an autologous patient with melanoma resulted in the objective regression of metastases (17). More recently, we cloned the gene TRP-1, or *gp75*, encoding the tumor antigen recognized by TIL586 in association with HLA-A31 (18). Interestingly, the gene product, gp75, was originally identified as an antigen recognized by IgG antibodies in the serum from a patient with matastatic melanoma (19). The gene was found to be expressed only in melanoma, normal melanocyte cell lines, and retina, but not in other normal tissues tested (18, 20). Therefore, this gene is a member of the second class of antigens including MART-1/Melan-A, gp100, and tyrosinase.

In this study we report the identification of the antigenic epitope from the TRP-1 gene. ExoIII/S1 deletion analysis allowed us to localize the epitope in a small DNA fragment but failed to identify the epitope from the normal gp75 protein using synthetic peptides. Surprisingly, the peptide recognized by TIL586 was derived from the gene product translated from an alternative open reading frame of the same gene. Substitution of the ATG start codon of this short open reading frame with ATC at nucleotides 294–296 resulted in a complete loss of the ability to stimulate cytokine release from TIL586. Cold-target inhibition ex-

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[1]*Abbreviations used in this paper:* ORF, open reading frame; TIL, tumor-infiltrating lymphocyte.

periments indicated that the identified epitope was capable of competing for T cell recognition with a naturally processed peptide present on the tumor cells. Six T cell clones generated from the TIL586 cell line were capable of recognizing 586mel tumor cells, 586EBV B cells pulsed with this peptide, and normal melanocytes in an HLA-A31–restricted fashion, also suggesting that the gene product encoded by the alternative open reading frame might be present in the tumor cells as well as the normal melanocytes. The biological significance and the mechanism by which the overlapping open reading frames are translated from a single cellular gene are discussed.

Materials and Methods

Chemicals and Reagents. The following chemicals and reagents were purchased from the sources indicated: RPMI 1640, AIM-V media, lipofectamine, G418 (GIBCO BRL, Gaithersburg, MD); the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA); anti–HLA-A31 mAb (One lambda, Canoga Park, CA); anti-IgM antibody conjugated with FITC (Vector Laboratories, Inc., Burlingame, CA).

CTLs and Cell Lines. TIL586 were isolated from the tumor specimen of a patient with metastatic melanoma and grown in medium containing IL-2 (6,000 IU/ml) (Cetus Corp., Emoryville, CA) for 32–60 d as previously described (17). TIL586 were predominantly CD8$^+$ T cells. TIL1200 were grown under the same conditions as described for TIL586. The T cell clones were generated by the limiting dilution method from the TIL586 cell line, and then they were expanded in AIM-V medium containing 6,000 IU/ml IL-2.

Melanoma cell lines 397mel, 397mel/A31, 586mel, 624mel, and EBV-transformed B cell lines 586EBV and 1510EBV were established in this laboratory and cultured in RPMI 1640 medium containing 10% FCS. Normal cultured melanocytes derived from infant foreskin (NHEM680, purchased from Clonetics Corp., San Francisco, CA) were cultured in melanocyte growth medium (MGM; Clonetics Corp.). The COS-7 cell line was provided by Dr. W. Leonard (National Institutes of Health, Bethesda, MD).

GM-CSF Secretion Assay. DNA transfection and GM-CSF assay were done as previously described (18). Briefly, 200 ng of plasmid DNA pools or clones and 50 ng of the HLA-A31 DNA were mixed with 2 μl of lipofectamine in 100 μl of DMEM for 15–45 min. The DNA–lipofectamine mixture was then added to the COS-7 (5 × 10^4) cells and incubated overnight. The following day, cells were washed twice with DMEM medium. TIL586 were added at a concentration of 10^5 cells/well in AIM-V medium containing 120 IU/ml of IL-2. After 18–24-h incubation, 100 μl of supernatant was collected, and GM-CSF was measured in a standard ELISA assay (R & D Systems Inc., Minneapolis, MN). For peptides, 586EBV, 1510EBV, and T2 cells were incubated with peptides at 37°C for 90 min and then washed three times with AIM-V medium containing 120 IU/ml of IL-2. TIL586 were added and incubated for an additional 18–24 h, and 100 μl of supernatant was collected for GM-CSF assay.

Exo III/S1 Deletion Constructions and PCR Fragments. To make a series of deletions, the pcDNA776 plasmid DNA was digested with XbaI and filled in with α-phosphorothioate deoxyribonucleotide triphosphates to block ExoIII nuclease digestion. The pcDNA776 plasmid is a derivative of the pcDNA3 vector containing a 2.4-kb DNA fragment of the *gp75* gene and a CMV promoter for directing transcription. The linearized DNA was

subjected to the second restriction enzyme XhoI digestion to generate one end sensitive to ExoIII. ExoIII nuclease/Mung bean nuclease deletion was performed according to the manufacturer's instructions (Stratagene Inc., La Jolla, CA). PCR amplification was performed at 94°C for 2 min followed by 25 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min. Primers gpN (5'-AGAATGAGTGCTCCTAAACTCCTCTCTCTGGG) and gp11B (5'-CATGTGAGAAAAGCTGGTCCCTCA) were used to generate the DNA fragment (1–667) and then cloned into the pCR3 expression vector to produce pPCR110. Plasmids pPCR210 and pPCR220 were pCR3 vectors containing DNA insertion fragments amplified by using primers gp-1 (5'-TGG-ATATGGCAAAGCGCACAACTC) and gp11B, gp-1, and gp22 (5'-TAAATGGAAATGTTCTCAAATTGTGGCGTG), respectively.

Cytotoxic Lysis Assays. Cytolytic assays were done as previously described (16). Briefly, the target cells were labeled with chromium for 90 min. After washing three times, the cells were incubated with peptides at a concentration of 1 μg/ml for 90 min. The cells were washed again, counted, and then mixed with TIL586 at the indicated E/T ratio. Chromium release was measured after 4 h incubation. The peptides were synthesized by a solid-phase method using a peptide synthesizer (model AMS 422; Gilson Co., Inc., Worthington, OH). Some peptides were purified by HPLC and were >98% pure. For titration of the ORF3P peptide recognized by TIL586, 586EBV B cells were incubated with various concentrations of the purified ORF3P peptide. Percentage of specific lysis was determined from the equation $(A - B)/(C - B) \times 100$, where A is lysis of 586EBV B cells by TIL586 in the presence of a peptide, B is spontaneous release from 586EBV B cells in the presence of the same peptide but in the absence of effector cells, and C is the maximum chromium release. Cold-target inhibition of cytolysis was performed using ^{51}Cr-labeled 586mel or 624mel cells as "hot" targets and 586EBV B and T2 cells pulsed with peptides as "cold" targets.

Site-directed Mutagenesis. For carrying out site-directed mutagenesis, we used mutated primers GPMUT1 (5'-GCCATGGGC-AGAGATGATCGGGAGGTCTGGCCCTTGCGCTTCTTC-AATAGGACAT$\underline{C}$TCACTGCAAC) and the non-mutated primer GPA1 (5'-AAGATCTGGGCCCGGACAAAGTGGTTCTTTTC) to generate a PCR fragment containing a mutation (G to C) at nucleotide 296. The wild-type DNA fragments were amplified by the use of primers GPF1 (5'-GAAGATCTGCCATGGGCA-GAGATGATCGGGAGGTCTG), GPE1 (5'-GAATTCGTTG TGT CCTGAGAAATTGCCGTTG), GPE2 (5'-GAATTCGAC-TATGAGAACCCTCTGGTCACAGGC),and GPA1, as indicated by arrowheads in Fig. 3 *A*. The purified PCR products were then cloned into the pCR3 expression vector. All plasmids containing PCR fragments were sequenced to confirm the orientation and nucleotide sequence.

Results

Localization of the Antigenic Peptide(s) Recognized by TIL586. To identify the antigenic epitope from gp75, we generated a series of nested deletions of *gp75* gene from the 3' end using ExoIII/S1 nuclease as well as additional DNA fragments from gp75 by PCR amplification (Fig. 1 *A*). We chose the pcDNA776 construct as a starting material for deletion studies. This clone, which was initially identified by a library screening, conferred the ability to stimulate cytokine release from TIL586. Since the goal of this study

was to identify the epitope recognized by TIL586, we used fragments of the truncated form of gp75, instead of full-length cDNA so that we could quickly locate the epitope in a relatively small DNA fragment. These deletion constructs were then transfected into COS-7 cells together with the pBK-CMV plasmid containing the HLA-A31 gene (18). After 24 h, the transfected COS-7 cells were tested to determine which construct could stimulate cytokine release by TIL586. A small truncated DNA fragment ranging from nucleotide 247 to 771, which lacked the normal gp75 initiation codon, retained the ability to stimulate GM-CSF release by TIL586, suggesting that the epitope recognized by TIL586 was located in the DNA fragment containing nucleotides from 247 to 771. Since there is an ATG start codon in a relatively good context of Kozak sequence (GATATGG) located at nucleotides 445–447 and in the same frame as gp75 open reading frame, we reasoned that the epitope recognized by TIL586 might be located in the region from nucleotide 445 to 771. Therefore, we constructed pPCR210 and pPCR220, which were derivatives of the pCR3 expression vector and contained an internal ATG codon in frame with gp75 (GATATGG) located at 445 bp as a start codon for translation of the truncated normal gp75 protein. However, neither pPCR210 nor pPCR220 conferred the ability to stimulate cytokine secretion from TIL586 after cotransfection of COS-7 with the HLA-A31 gene (Fig. 1 B), suggesting that the epitope was located upstream of these fragments. Therefore, an additional plasmid pD776A was constructed that contained the nucleotide sequence from 247 to 442 and did not have any ATG codon in the same frame as gp75, but did contain two ATG codons in different open reading frames relative to gp75. Surprisingly, this plasmid strongly stimulated cytokine release from TIL586 when cotransfected with A31 cDNA into COS-7 cells. The plasmid pPCR110 containing the authentic start codon of gp75 stimulated severalfold lower cytokine release than did pDel 5 or pD776A when cotransfected with the HLA-A31 gene (Fig. 1 B). These results suggested that the epitope(s) recognized by TIL586 were located in the region from nucleotides 247 to 442.

Although this region (nucleotides 247–442) did not have any ATG start codon in the normal gp75 open reading frame, initiation of translation from non-ATG codons such as ACG, CTG, and GTG had been reported in some cases (21–23). To identify the epitope in this region, we made synthetic peptides based on the peptide binding motif of HLA-A31 (hydrophobic residue at position 2 and positively charged residue at the COOH terminus) (Fig. 2) (24). The majority of the peptides selected for this study were nonamers, although some were 10 mers and 11 mers. We pulsed these peptides onto 586EBV B cells and tested the ability of these cells to stimulate cytokine release by TIL586 (Table 1). One peptide, AACDQRVLIVRR, very weakly induced GM-CSF release from TIL586. However, this peptide failed to sensitize peptide-loaded 586EBV B for lysis by TIL586 (Table 1). Because this peptide weakly stimulated cytokine release from TIL586 only when incubated with 586EBV B cells at high concentrations (>1 µg/
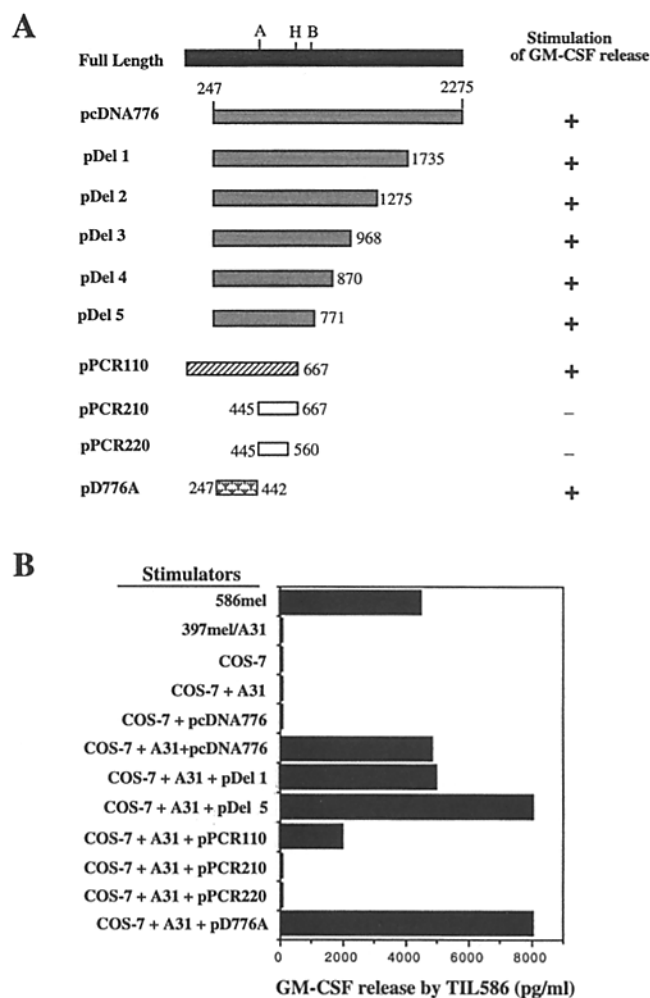
Figure 1. Location of the *gp75* nucleotide sequence coding for the antigenic peptides recognized by TIL586. (*A*) The full-length cDNA comprising the 1584-bp open reading frame of gp75 is shown. Nucleotides are numbered from the start codon, which translates into a protein consisting of a leader sequence and the mature gp75. pcDNA776 is a partial cDNA of *gp75* that lacks the first 246 nucleotide coding region, and it was isolated from a cDNA library using an assay based on its ability to stimulate GM-CSF secretion by TIL586 when cotransfecting COS-7 along with the HLA-A31 gene. A series of deletion constructs and PCR DNA fragments were made. pD776A is a derivative of pcDNA776 after digestion with the ApaI restriction enzyme. (*B*) GM-CSF release by TIL586. GM-CSF secretion by TIL586 was measured after coculture with COS-7 cotransfected with the DNA fragments shown in *A* and the HLA-A31 gene. Control stimulator cells include 586mel, 397mel/A31[+], COS-7 alone, and COS-7 transfected either with HLA-A31 or pcDNA776 cDNA.

ml) and did not sensitize the target cells for lysis by TIL586 even at 10 µg/ml of peptide concentration (data not shown), it may not represent the predominant T cell epitope recognized by TIL586.

To further define the region containing the predominant T cell epitope, we constructed two additional plasmids containing PCR fragments amplified by primers GPF1, GPE1, and GPE2, respectively (Fig. 3 *A*). As shown in Fig. 3 *B*, both plasmids conferred the ability to stimulate cytokine release by TIL586 in association with HLA-A31, suggest-

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ATGAGTGCTCCTAAACTCCTCTCTCTGGGCTGTATCTTCTTCTTCCCCTTGCTACTTTTTCAG  60
ORF1     M  S  A  P  K  L  L  S  L  G  C  I  F  F  P  L  L  L  F  Q
         CAGGCCCGGGCTCAATTCCCAAGACAGTGTGCCACTGTTGAGGCTTTGAGAAGTGGTATG  120
ORF1     Q  A  R  A  Q  F  P  R  Q  C  A  T  V  E  A  L  R  S  G  M
         TGTTGCCCAGACCTGTCCCCTGTGTCTGGGCCTGGGACAGACCGCTGTGGCTCATCATCA  180
ORF1     C  C  P  D  L  S  P  V  S  G  P  G  T  D  R  C  G  S  S  S
         GGGAGGGGCAGATGTGAGGCAGTGACTGCAGACTCCCGGCCCCACAGCCCTCAGTATCCC  240
ORF1     G  R  G  R  C  E  A  V  T  A  D  S  R  P  H  S  P  Q  Y  P
         CATGATGGCAGAGATGATCGGGAGGTCTGGCCCTTGCGCTTCTTCAATAGGACATGTCAC  300
ORF1     H  D  G  R  D  D  R  E  V  W  P  L  R  F  F  N  R  T  C  H
ORF2                M  I  G  R  S  G  P  C  A  S  S  I  G  H  V  T
ORF3                                                    M  S  L


         TGCAACGGCAATTTCTCAGGACACAACTGTGGGACGTGCCGTCCTGGCTGGAGAGGAGCT  360
ORF1     C  N  G  N  F  S  G  H  N  C  G  T  C  R  P  G  W  R  G  A
ORF2     A  T  A  I  S  Q  D  T  T  V  G  R  A  V  L  A  G  E  E  L
ORF3     Q  R  Q  F  L  R  T  Q  L  W  D  V  P  S  W  L  E  R  S  C


         GCCTGTGACCAGAGGGGTTCTCATAGTCAGGAGAAATCTTCTGGACTTAAGTAAAGAAGAA  420
ORF1     A  C  D  Q  R  V  L  I  V  R  R  N  L  L  D  L  S  K  E  E
ORF2     P  V  T  R  G  F  S  STOP
ORF3     L  STOP


                             ApaI
         AAGAACCACTTTGTCGGGCCCTGGATATGGCAAAGCGCACAACTCACCCT...ATATGA  1584
ORF1     K  N  H  F  V  R  A  L  D  M  A  K  R  T  T  H  P
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Figure 2. The nucleotide, amino acid sequence, and open reading frames of the gp75 gene. The partial nucleotide and amino acid sequences of the first 157 amino acids were shown from the start codon for translation of ORF1 (gp75). The DNA fragment that conferred the ability to stimulate GM–CSF release from TIL586 is underlined. Two putative start codons, ATG (254–256) and ATG (294–296), are in bold and may result in the translation of ORF2 and ORF3, respectively. The peptide sequence recognized by TIL586 from ORF3 is in bold and underlined.

ing that the epitope recognized by T cells was encoded within an 82-nucleotide sequence between bases 247 and 329 of the *gp75* cDNA. 28 overlapping peptides (9 mers or 10 mers) in the gp75 open reading frame (ORF) 1 were synthesized based on the amino acid sequence of gp75 in

this region, but none were found to stimulate GM–CSF release or sensitize 586EBV B cells for lysis by TIL586 (data not shown).

Antigenic Peptides Resulted from Translation of an Alternative Open Reading Frame of the gp75 Gene. The failure to identify an epitope recognized by TIL586 in this small region suggested to us that alternative open reading frames might be translated. Two ATG codons in the relatively good context were present in this region (nucleotides 247–442) in different open reading frames from ORF1 (Fig. 2). Translation from the first ATG (251 GAGATGA257) resulted in a open reading frame encoding 45 amino acids open reading frame 2 (ORF2), whereas translation starting from the ATG located between nucleotides 294 and 296 (GAC-ATGT) generated a 24–amino acid gene product open reading frame 3 (ORF3) (Fig. 2). Three peptides derived from ORF2 and two peptides from ORF3 were selected and synthesized on the basis of the HLA-A31 binding motif (Table 2 and Fig. 2). Surprisingly, one peptide, MSL-QRQFLR (designated as ORF3P), which derived from ORF3, was strongly recognized by TIL586 when pulsed onto 586EBV B cells (Table 2). The recognition of the ORF3P peptide (MSLQRQFLR) by TIL586 was observed only when the peptide was pulsed onto autologous 586EBV B cells and 1510EBV B (A31+) cells, but not when peptide was loaded onto T2 (non–HLA-A31) cells (Fig. 4 *A*), suggesting that recognition of this peptide by TIL586 was HLA-A31 restricted. The peptide mass of ORF3P was confirmed by mass spectrometry. As shown in Fig. 4 *B*,

Table 1. *Screening of Synthetic Peptides with Reactivity to TIL586*

Target cells pulsed with peptide	TIL586	
	GM–CSF release	Percent specific lysis (E/T ratio 20:1)
Peptides from ORF1 (gp75)		
586EBV + peptide DDREVWPLR	<50	<10
586EBV + peptide VWPLRFFNR	<50	<10
586EBV + peptide SGHNCGTCR	<50	<10
586EBV + peptide CGTCRPGWR	<50	<10
586EBV + peptide ACDQRVLIVR	<50	<10
586EBV + peptide ACDQRVLIVRR	<50	<10
586EBV + peptide LWDVPSWLER	<50	<10
586EBV + peptide AISQDTTVGR	<50	<10
586EBV + peptide AACDQRVLIVR	250	<10
586EBV + peptide DQRVLIVRR	<50	<10
586EBV + peptide IVRRNLLDLSK	<50	<10
586EBV + peptide LSKEEKNHFVR	<50	<10
586EBV + none	<50	<10
586mel + none	>5,000	<45

586EBV cells were incubated with individual peptide at a concentration of 1 μg/ml for 90 min. GM–CSF release was measured after coincubation of peptide-loaded 586EBV cells with TIL586. GM-CSF secretion by TIL586 alone without stimulators was subtracted. 586EBV was an EBV-transformed B cell line expressing HLA-A31. Cytotoxic lysis of peptide-pulsed 586EBV by TIL586 was done in a 4-h ^{51}Cr release assay.
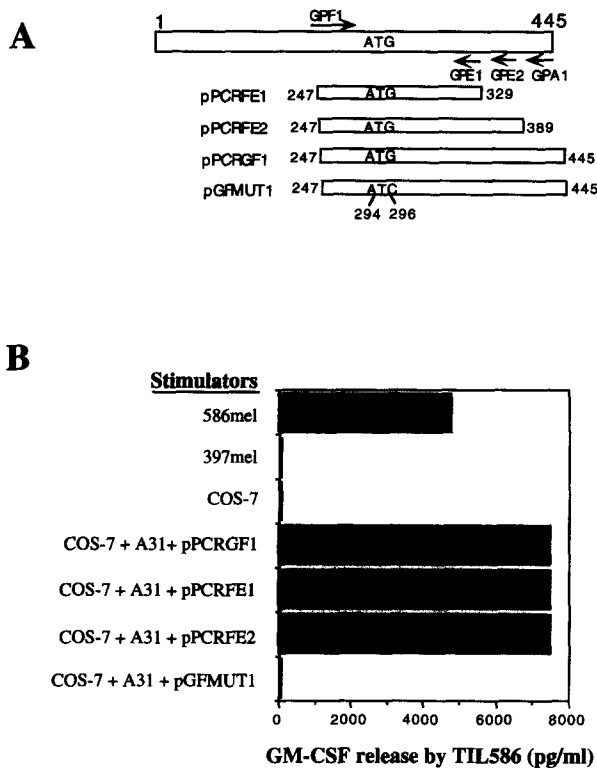
A

B

Figure 3. An antigenic peptide and translation of an alternative open reading frame. (*A*) Location and length of PCR fragments amplified by PCR. DNA fragments were obtained by PCR amplification and were then cloned into the pCR3 expression vector. Substitution of ATG at positions 294–296 with ATC was made as described in Materials and Methods. (*B*) Test of DNA fragments and mutation constructs to stimulate cytokine release from TIL586. GM-CSF release assay was done as in Fig. 1.

TIL586 lysed 586EBV B cells pulsed with the ORF3P peptide, but failed to lyse 586EBV B cells pulsed with an irrelevant peptide, which met the criterion of the peptide-binding motif of HLA-A31 or T2 cells pulsed with the ORF3P peptide. Sensitization for lysis by the peptide showed maximal effect at 100 nM, though lytic activity was detected even at 1 nM of peptide concentration (Fig. 4 *C*). TIL586 did not recognize either peptides MSLQRQFLR**T** or SLQRQFLRT, or modified peptides containing substitution of anchor residues at positions 2, 6 and 9 such as M**L**LQRQFLR, M**R**LQRQFLR, MSLQR**L**FLR, MSLQRQFL**E**, or MSLQRQFL**K** (Table 2). TIL586 only recognized the peptide MA**L**QRQFLR containing a substitution of Ser with Ala at position 2 compared with the peptide MSLQRQFLR (Table 2).

Translation of ORF3 Is Necessary for Generating the Antigenic Peptide. Since there was a stop codon TAG (288–290) located in the six nucleotides upstream of the ATG start codon of ORF3 (294–296) (Fig. 2), it was unlikely that the ORF3P peptide resulted from a frameshift. DNA sequence analysis also confirmed that there was no deletion or insertion in the upstream region. To investigate if the ATG located at nucleotides 294–296 played an important role in translating the 24–amino acid product, we mutated AT**GT**

(294–297) to AT**CT** (294–297) to eliminate the translation of ORF3, which would result in a change of Cys (UGU) to Ser (UCU) in ORF1 (gp75) (Fig. 3 *A*). A plasmid containing the mutated gene (pGFMUT1) was tested for its ability to confer recognition by TIL586 when cotransfected into COS-7 along with the HLA-A31 cDNA. Fig. 3 *B* showed that the mutated gene completely lost the ability to stimulate GM-CSF release by TIL586. This observation indicated that ATG in ORF3 in nucleotide positions 294–296 was required for translation of the 24–amino acid product, and therefore was essential for generating the T cell epitope recognized by TIL586.

Since the Met (ATG) is in position 1 of the peptide epitope, and the mutation of ATG to ATC at nucleotides 294–296 resulted in a change of Met to Ile in position 1 of the peptide, we further investigated the possibility that the loss of recognition of the mutated gene by TIL586 could be due to the loss of the ability of the mutated peptide to bind to MHC class I molecules. A synthetic peptide (ISLQRQFLR) with the same amino acid sequence as that encoded by the mutated gene was made and tested for recognition by TIL586. It was found that the synthetic mutated peptide was still recognized by TIL586 at concentrations comparable to that of the wild-type peptide (data not shown). Furthermore, when the same mutation was introduced into the full-length cDNA, no reactivity to TIL586 was observed, whereas the wild-type cDNA was capable of stimulating cytokine release from TIL586 at a level similar to pPCR-110 (data not shown). This is in agreement with the deletion data, indicating that TIL586 did not recognize peptide(s) in other regions of the gene. These results suggested that the loss of recognition of the mutated gene containing an ATG to ATC substitution at nucleotides 294–296 by T cells was due to inhibition of translation initiation of ORF3.

Recognition of the Antigenic Peptide on Tumor Cells As Well As Melanocytes. To address the question of whether TIL586 recognized a naturally processed peptide similar or identical to the ORF3P peptide on the tumor cells, we examined the ability of the ORF3P peptide–pulsed 586EBV B cells to inhibit lysis of ^{51}Cr-labeled 586mel in a cold-target inhibition assay. Significant inhibition of lysis of ^{51}Cr-labeled 586mel was observed by 586EBV B cells pulsed with the ORF3P peptide but not by 586EBV B cells pulsed with irrelevant peptide or T2 cells pulsed with the ORF3P peptide (Fig. 5 *A*), indicating that this peptide epitope was capable of competing with a naturally processed peptide on tumor cells for T cell recognition. As predicted, the ORF3P-loaded 586EBV B cells did not inhibit lysis of target 624mel by TIL1200, which recognizes the gp100 antigen in the HLA-A2 context, compared with 586EBV B alone (Fig. 5 *B*). To further test if T cell clones can recognize both the ORF3P peptide–pulsed 586EBV B cells and tumor cell lines, T cell clones were generated from the TIL586 cell line by limiting dilution (1 cell/well in 96-well round bottom microplate) and further expanded in culture. Six T cell clones were capable of recognizing 586mel tumor cells, 586EBV
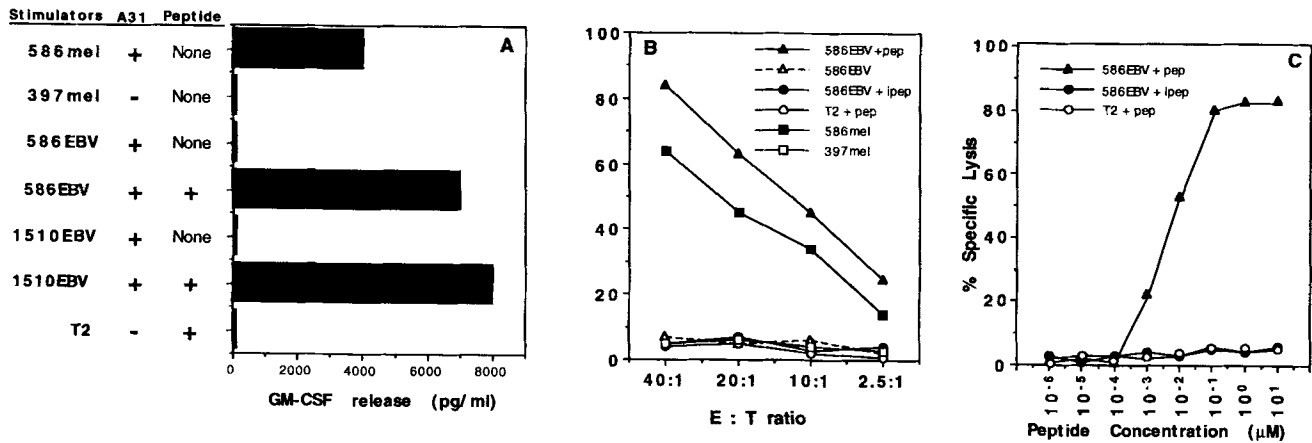
Figure 4. Characterization of the antigenic peptide recognized by TIL586. (A) GM-CSF release by the HLA-A31–restricted TIL586 when coincubated with various stimulators. Transfection and cytokine assays were performed as in Fig. 1. 586mel and 397mel were included as positive and negative controls for the reactivity of TIL586. The ORF3P peptide was incubated with 586EBV (A31+) and T2 (non-A31) cells at a concentration of 1 μg/ml for 90 min. Stimulation of GM-CSF secretion by TIL586 significantly increased when coincubated with autologous 586EBV and allogeneic 1510EBV (A31+) cells pulsed with peptide ORF3P, but not when coincubated with either 586EBV alone or T2 (non-A31) cells loaded with the ORF3P peptide. (B) Cytotoxic lysis of the target cells by TIL586. 586mel (—■—) and 397mel (—□—) were used as positive and negative controls, respectively. 586EBV B cells were incubated with ORF3P (pep) (—▲—), with an irrelevant peptide (ipep) (—●—), or without peptide (—△—), and T2 cells were pulsed with ORF3P (—○—) as marked. After incubation, TIL586 was added and mixed with the target cells. Cytolytic activity of TIL586 was measured in a 4-h ^{51}Cr release assay. (C) Titration of the peptide concentration to sensitize the target cells for lysis by TIL586. 586EBV cells were separately incubated with serial dilutions of ORF3P (pep) (—▲—) or irrelevant peptides (ipep) (—●—) and T2 cells with the ORF3P peptide (—○—) for 90 min. The cytolytic activity of TIL586 was evaluated in a 4-h ^{51}Cr release assay at an E/T ratio of 40:1.

B cells pulsed with the ORF3P peptide, and HLA-A31 positive melanocytes, but not 397mel/A31 + or 586EBV B cells alone. Representative data are shown in Fig. 6. These results suggested that T cell clones probably recognized a naturally processed peptide either similar or identical to the ORF3P peptide on tumor cells and normal melanocytes.

Since there is a 40–45% amino acid sequence identity of gp75 to tyrosinase, gp100, and TRP-2, we tested the possibility that the peptide recognized by the T cell clones was not derived from gp75, but from one of these other proteins. We thus transfected COS-7 cells with HLA-A31 plus either tyrosinase, gp100, or TRP-2 cDNAs, and found that none could be recognized by the six T cell clones, whereas the COS-7–transfected HLA-A31 and gp75 cDNA stimulated GM-CSF release from these clones (data not shown). A computer database search also indicated that no known proteins, including tyrosinase, gp100, and TRP-2, in the available database contained amino acid sequences with the peptide-binding motif of HLA-A31 and significant similarity to the peptide epitope recognized by TIL586.

Discussion

Several antigenic T cell epitopes derived from the normal open reading frame of the corresponding nonmutated shared melanoma antigens such as tyrosinase, MART-1/Melan-A, and gp100 have been recently identified (16, 25–27). In this study, we demonstrated that the antigenic peptide recognized by TIL586 was derived from a second gene product of the gp75 gene. To our knowledge, this is the first example that T cells recognize an antigenic peptide re-

sulting from the translation of an overlapping open reading frame of the same gene and the only example in eukaryotic cells that two completely different proteins and/or peptides can be translated from overlapping open reading frames of a single cellular gene. The ORF3 of the gp75 gene encodes a short protein of 24 amino acids whose normal function, if any, is unknown. The antigenic peptide recognized by TIL586 is encoded by the sequence located immediately behind the ATG (294–296) start codon of the alternative open reading frame.

Although gp75 shares a 40–45% amino acid sequence identity to tyrosinase, gp100, and TRP-2, cotransfection of HLA-A31 and tyrosinase, gp100, or TRP-2 cDNAs, respectively, into COS-7 cells failed to stimulate GM-CSF release from T cell clones derived from TIL586. A database search did not reveal any proteins that had the HLA-A31 peptide binding motif and significant sequence homology to the peptide epitope recognized by TIL586 and its derived T cell clones. In addition, previous studies showed that melanoma transfectants (gp75+/A31+) conferred the ability to stimulate GM-CSF release from TIL586, but gp75⁻ melanoma transfectants (gp75⁻/A31+) did not (18). Similar results were obtained with additional melanoma cell lines (gp75⁻/A31+). These results suggested that it was unlikely that TIL586 recognized the epitope peptide derived from other known genes. Since the ORF3P peptide was the only epitope identified from the gp75 gene and was recognized by six T cell clones derived from TIL586, this peptide may be identical or similar to the naturally processed peptide on tumor cells and melanocytes. This was further supported by cold-target inhibition experiments, since this peptide was capable of competing for T cell rec-

Table 2. *Identification of Antigenic Peptides with Reactivity to TIL586*

Target cells pulsed with peptide	TIL586	
	GM-CSF release	Percent specific lysis (E/T ratio 20:1)
Experiment A		
Peptides derived from ORF2		
586EBV + peptide ISQDTTVGR	<50	<10
586EBV + peptide AISQDTTVGR	<50	<10
586EBV + peptide AGEELPVTR	<50	<10
Peptides derived from ORF3		
586EBV + peptide LWDVPSWLER	<50	<10
586EBV + peptide MSLQRQFLR	>8,000	60
586EBV + none	<50	<10
586mel + none	>5,000	47
Experiment B		
Modified peptides of MSLQRQFLR		
586EBV + peptide MSLQRQFLR	>8,000	63
586EBV + peptide MSLQRQFLRT	<50	<10
586EBV + peptide SLQRQFLRT	<50	<10
586EBV + peptide MALQRQFLR	>5,000	54
586EBV + peptide MLLQRQFLR	<50	<10
586EBV + peptide MRLQRQFLR	<50	<10
586EBV + peptide MSLQRLFLR	<50	<10
586EBV + peptide MSLQRQFLE	<50	<10
586EBV + peptide MSLQRQFLK	<50	<10
586EBV + none	<50	<10
586mel + none	>5,000	48

Conditions for peptide incubation with 586EBV B cells and GM-CSF release assay were the same as described in Table 1. GM-CSF secretion by TIL586 alone without stimulators was subtracted. Modified peptides were made by substitution of amino acid at positions 2, 6, and 9 relative to MSLQRQFLR. Cytotoxic lysis of peptide-pulsed 586EBV by TIL586 was done in a 4-h ^{51}Cr release assay.

ognition with a natural peptide on tumor cells. However, we could not exclude the possibility that these T cells may cross-react with another peptide derived from a distinct protein on tumor cells and melanocytes, since we have not directly isolated the ORF3P peptide from tumor cells.

It was reported that T cell–epitope peptides derived from the frameshift of the mutated adenomatosis polyposis coli gene in colon cancer were recognized by CTLs generated from vaccinated BALB/c mice (28). The results shown in Fig. 3 indicated that the ATG at nucleotides 294–296 was required for translation of the 24–amino acid product, which, in turn, was processed to the antigenic peptide recognized by TIL586. TIL586 still recognized the mutated synthetic peptide pulsed on 586EBV B cells, but not the mutated gene when cotransfected into COS-7 cells with HLA-A31 cDNA, indicating that the loss of recognition of the mutated (ATG to ATC) gene by TIL586 resulted from elimination of translation of the ORF3 product. These results plus DNA sequence analysis ruled out the possibility that the antigenic peptide recognized by TIL586 was derived from the frameshift product of gp75. It is possible, therefore, that multiple

peptides or proteins are often translated from overlapping open reading frames of a single eukaryotic gene, but that means to detect these alternate products have not been available. The exquisite sensitivity of T cells which can detect naturally processed peptides may allow one to reveal many other examples of this phenomenon.

The mechanism by which the overlapping ORF3 is translated is currently unclear. Although examples have been reported of cellular mRNAs that initiate at more than one AUG codon (29, 30), and that, in some rare cases, initiate at both AUG and non-AUG codons such as CUG to generate NH_2-terminally extended identical sequences (21, 23), the use of overlapping open reading frames (i.e., translating two completely dissimilar peptides) from a single eukaryotic cellular mRNA has to our knowledge never been described. Several examples of translation of overlapping open reading frames from a single mRNA have been described, but they are exclusively limited to viral genes (31–34). The detection of the products of overlapping open reading frames in viral genes has been possible because of the existence of reactive antibodies in the sera of virally infected
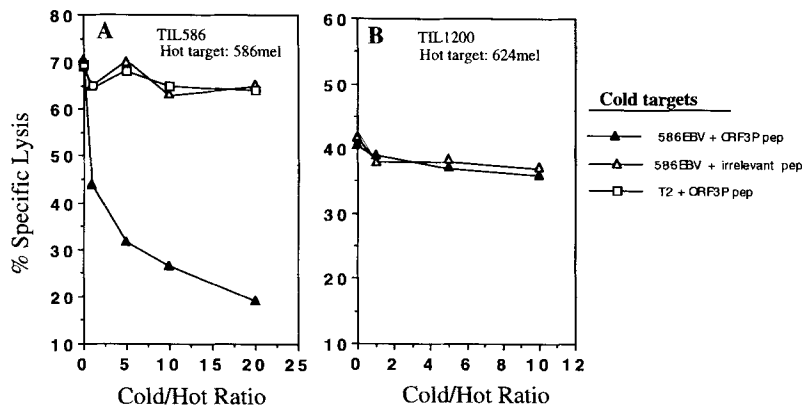
Figure 5. Inhibition of lysis of ³¹Cr-labeled target cells by nonlabeled 586EBV cells loaded with the ORF3P peptide. (A) 586mel cells were labeled with ^{51}Cr for 90 min as a "hot" target. 586EBV cells pulsed with ORF3P (—▲—), with irrelevant peptide (–△–), and T2 cells loaded with ORF3P (—□—) were used as "cold" target cells. After washing, hot and cold target cells were counted again and mixed at the cold/hot ratios of 1, 5, 10, and 20:1. TIL586 was added at an effector/hot target (E/T) ratio of 20:1. ^{51}Cr release was measured after a 4-h incubation. (B) Lysis of ^{51}Cr-labeled 624mel (hot target) by TIL1200, which recognized gp100, was not inhibited by 586EBV cells pulsed with ORF3P (—▲—) compared with 586EBV cells pulsed with an irrelevant peptide (–△–). Cold and hot target cells were mixed at the indicated ratios. TIL1200 was added at an effector/hot target (E/T) ratio of 30:1. Cytolytic activity of TIL1200 was evaluated in a 4-h ^{51}Cr release assay.

hosts. In our case, we used a T cell assay to identify the epitope peptide recognized by T cells. This approach is very different from and more sensitive than conventional Western blots or immunoprecipitation analyses (35). Although there are five ATG codons between the authentic start codon of gp75 protein and the start codon of ORF3, the construct pPCR110 covering the NH$_2$-terminal part of ORF1 (gp75) and the entire ORF2 and ORF3 (nucleotides 1–667) still retained the ability to stimulate cytokine release from TIL586. The level of stimulation by pPCR110, however, was severalfold lower than that stimulated by the 5' truncated (lacking the first 246 nucleotides) form of gp75 (Fig. 1, A and B), suggesting that the upstream ATG codons may have partially inhibited the expression of ORF3. Several factors have made it possible to detect the expression of the ORF3 product in this system. First, the upstream ATG codons preceding the ATG start codon of ORF3 did not appear to be in the optimal context, which may allow use of the downstream ATG as start codons by the leaky scanning model. Second, the relatively high expression of transfected genes in COS-7 cells and the availability of the T cell assay as an extremely sensitive means may allow us to detect very low levels of the translated products.

Interestingly, the ORF3 product was detected by T cells in the tumor cells as well as normal melanocytes (Fig. 6), strongly suggesting that the ORF3 protein was not a gene product resulting from genetic alterations in tumor cells. In previous studies, it was shown that TIL586 recognized multiple tumor cell lines (gp75$^+$/A31$^+$) tested, suggesting that TIL586 recognizes a nonmutated, shared tumor antigen (18). Since the gp75 gene is highly expressed in melanomas based on Northern blot and PCR analyses (18), and its gene product gp75 protein is the most abundant intracellular glycoprotein expressed in melanoma cells and melanocytes (36–38), it is not surprising that the T cell clones recognized the ORF3P peptide when pulsed onto 586EBV B cells (A31$^+$), and also recognized melanoma (gp75$^+$/A31$^+$) as well as A31$^+$ melanocytes, but not gp75$^-$/A31$^+$ melanoma cells or ORF3P pulsed on non-A31 T2 cells. An-

other possibility to explain the peptide expression in tumor cells and melanocytes is that ORF3 may be translated from a separate mRNA transcript(s) generated by an alternative splicing of gp75 mRNA or a different promoter. To our knowledge, mRNA transcripts generated by alternative splicing are translated into isoform proteins by use of the same open reading frames. In our case, however, a separate transcript may be generated and used as a template for trans-
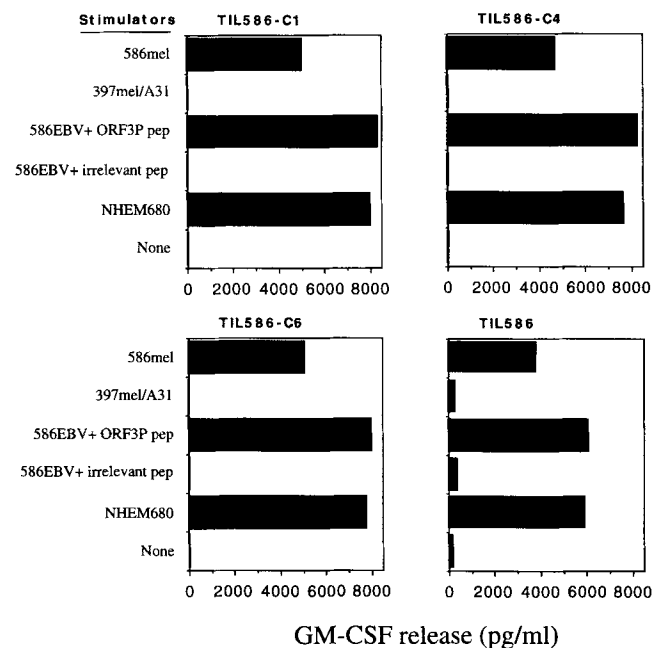

GM-CSF release (pg/ml)

Figure 6. Recognition of the antigenic peptide by T cell clones from the TIL586 cell line. T cell clones were generated by limiting dilution (1 cell/well) from the TIL586 cell line. T cell clones were further expanded in AIM-V medium containing 6,000 IU/ml IL-2. 586EBV B cells were pulsed with the ORF3P peptide or irrelevant peptide for 90 min at 37°C. After washing three times, T cell clone or TIL586 cells were added and coincubated for an additional 18–24 h. For 586mel, 397mel/A31$^+$ tumors, and melanocyte NHEM680 cells, 10^5 cells/well were incubated with 10^5 cells of T cell cones, TIL586-C1, TIL586-C4 and TIL586-C6, or TIL586 for 18–24 h. GM-CSF assay was performed as described in Fig. 1.

lation, but a completely different open reading frame relative to gp75 was used to translate the ORF3 product. The complete structural organization of gp75 is presently unknown. Further experiments are needed to clarify the mechanisms for the translation of the ORF3 protein. Nevertheless, the possibilities mentioned above are not mutually exclusive.

Several years ago, the pepton hypothesis was proposed by Boon and Van Pel (39) to explain the observation that a short piece of promoterless subgenic DNA fragments transfected into P1 tumor cells directed the synthesis and surface expression of P91A tum⁻ antigen. This hypothesis, however, requires an unidentified new RNA polymerase and other factors to direct transcription and states that peptons are expressed in all tissues (39, 40). Although the pepton hypothesis explains a surprising observation, it fails to explain how an alternative open reading frame was translated

from *gp75* mRNA, and the tissue specificity of this gene expression. The *gp75* gene expression requires a classic RNA polymerase II promoter such as CMV promoter and the normal gp75 promoter.

In summary, the *gp75* gene encodes two gene products, the 24–amino acid peptide of ORF3, which was processed to an antigenic peptide recognized by T cells, and the gp75 protein, which was previously identified as an antigen by IgG antibodies in the sera from a patient with melanoma. The infusion of TIL586 plus IL-2 into an autologous patient with metastatic melanoma resulted in the objective regression of tumor (17). These results suggest that the MSLQRQFLR peptide derived from ORF3 and recognized by TIL586 is a tumor rejection antigen. The products of this gene may be promising candidates for developing immunotherapeutic strategies for the treatment of patients with melanoma.

Note added in proof: The use of alternative open reading frames for translating two gene products, p16[INK4a] and p19[ARF], from the p16 tumor suppressor gene has recently been reported (41).

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