ABSTRACT

CD4+ T-cell responses against human tumor antigens are a potentially critical component of the antitumor immune response. Molecular methods have been devised for rapidly identifying MHC class II-restricted tumor antigens and elucidating the recognized epitopes. We describe here the identification of neo-poly(A) polymerase (neo-PAP), a novel RNA processing enzyme overexpressed in a variety of human cancers, by screening a melanoma-derived invariant chain fusion cDNA library with tumor-reactive CD4+ T lymphocytes. A cryptic nonmutated HLA-DRβ1*0701-restricted neo-PAP epitope was processed through the endogenous MHC class II pathway. A unique point mutation effected a nonconservative substitution of a leucine for a proline residue at a structurally important site in neo-PAP that was remote from the recognized peptide, revealing a normally silent epitope for immune recognition. Genetic aberrations such as the described point mutation can have unexpected immunological consequences, in this case leading to immune recognition of a distant normal self epitope.

INTRODUCTION

The CD4+ T lymphocyte subset, characterized chiefly as immune regulators but also important in performing effector and memory functions, was demonstrated to be a critical component of antitumor immune responses in animal models concurrent with observations that tumor-specific CD4+ T cells reside in cancer patients (1). To understand the mechanisms by which human CD4+ T cells can interact with cancer cells, efforts have focused on identifying MHC class II-restricted tumor-associated Ags recognized by these lymphocytes. De novo Ag identification required the development of novel methods for biochemical purification (2, 3) or molecular cloning (4). With reliable methods now in hand, it is possible to learn more about the kinds of tumor Ag recognized by the “helper” arm of the cellular immune response. The limited number of MHC class II-restricted human tumor Ag identified thus far can be grouped into the general categories of nonmutated tissue lineage-specific Ag (e.g., tyrosinase and gp100 for melanoma), cancer/testis Ag (e.g., MAGE-3, NY-ESO-1), and mutated Ag (e.g., TPI, CDC27; Refs. 3 and 4). Although commonly expressed tumor Ag including the lineage-specific and cancer/testis Ag are candidates for clinical development as immunotherapeutic agents, the characterization of uniquely mutated Ag has elucidated mechanisms by which normally silent tumor-associated proteins can become visible to the immune system. In addition to instances in which the mutation itself can constitute an MHC class II-restricted neo-epitope (TPI; Ref. 3), mutations can also have indi-
inactivated human AB serum, with IL2 120 IU/ml (Chiron Corp., Emeryville, CA). Twenty-four h later, culture supernatants were harvested and assessed for the presence of secreted GM-CSF by ELISA (R&D Systems, Minneapolis, MN).

**DNA Sequencing.** DNA sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer/ABI, Foster City, CA). Sequences were determined with an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). Database searches for nucleotide and deduced amino acid sequence similarities were performed with the BLAST program.4

**Peptide Synthesis and T-Cell Recognition Assays.** Peptides were synthesized using standard N-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry and analyzed for sequence and purity as described (9). To test peptides for recognition by CD4+ TIL 1087, 1087-EBV-B cells were dispensed into flat-bottomed 96-well plates at 1.5 × 10^4 cells/well in RPMI 1640 + 10% human AB serum. Peptides dissolved in PBS + 1% DMSO were added directly to the wells at various concentrations, for a 20-h incubation at 37°C. The following day, TILs were added at 2 × 10^5 cells/well in the presence of IL2 (120 IU/ml) for another overnight incubation. Then, culture supernatants were microfiltered with a 0.22 µm filter (Millipore) and centrifuged at 13,000 rpm for 15 min. Supernatants were then assayed for the presence of secreted GM-CSF by ELISA (R&D Systems, Minneapolis, MN).

**Immunofluorescent Staining and Confocal Microscopy.** To study the intracellular localization of wild-type and mutant neo-PAPs, epitope tags permitting mAb-mediated detection were engineered onto the NH2- and COOH-termini of these molecules using PCR. The forward oligonucleotide PCR primer contained an EcoRV restriction site and a 30-bp sequence encoding an NH2-terminal influenza virus HA epitope tag in frame with the 5’ coding sequence of neo-PAP, and has been described elsewhere (6). The reverse primer, 5’-TTATGGGGCCGCTACTTGATCAACATCCTTTGAGTCCTCATTAAGGGTCCAGTGC3’, contained a NotI restriction site (underlined) and stop codon (bold), followed by a sequence encoding the 8-mer FLAG epitope (italics; Ref. 3) in-frame with a sequence encoding the COOH terminus of neo-PAP. DNA templates for PCR were cDNA clones encoding full-length wild-type or mutant neo-PAPs that had been amplified from 1087-mel cells, ligated into the pcR-BluntII-TOPO vector (Invitrogen), and completely sequenced (6). The new PCR products were ligated into the eukaryotic expression plasmid pEAK8, and their DNA sequences were confirmed. The plasmids pEAK8/Ha-neoPAP-FLAG wild type or mutant were transfected into exponentially growing HeLa cells (human cervical cancer; American Type Culture Collection, Manassas, VA) adherent to glass coverslips in 24-well culture plates using the LipofectAMINE Plus reagent (Invitrogen). Twenty h later, cells were fixed, permeabilized, and blocked as described previously (6) and then were stained with 5 µg/ml FITC-conjugated rat mAb directed against the HA epitope (clone 3F10; Roche Molecular Biochemicals, Indianapolis, IN) for 1 h at room temperature. After three washes with PBS, the cells were stained with 20 µg/ml anti-FLAG M2 murine mAb (Sigma, St. Louis, MO) followed by three more washes, and then cells were counterstained with 20 µg/ml Texas Red-X conjugated goat antimouse mAb. Antibodies were diluted in PBS containing 2% goat serum and 2% BSA. Coverslips were washed three times in PBS and mounted onto microscope slides using GelMount (Bionema Corp., Foster City, CA). Stained cells were examined on a Zeiss Axioplan microscope with a ×100/1.4 oil immersion objective, and confocal images were generated using a Zeiss LSM510 scanning laser microscope with laser excitation at 488 and 543 nm. Multitracking was used to prevent bleed-through between the fluoroscein and Texas-red channels. The total magnification was ×980.

**RESULTS AND DISCUSSION**

Identification of the Ag and Epitope Recognized by CD4+ TIL 1087. CD4+ T cells infiltrating a lymph nodal melanoma metastasis were cultured in the presence of IL2 but without Ag restimulation for up to 100 days. They specifically recognized whole autologous tumor cells (fresh or cultured) expressing MHC class II molecules, as manifested by cytolysis or by release of the cytokines GM-CSF and IFN-γ (8). Autologous EBV-B cells were not recognized, nor were allogeneic melanomas collectively sharing all of the MHC class II molecules expressed by TIL 1087 (see “Materials and Methods” above), which suggested that the recognized Ag was tumor specific and might be mutated. Significantly, 1087-EBV could not function as antigen presenting cells (APC) for exogenously pulsed lysates of 1087-mel cells, nor could allogeneic EBV-B cells that shared MHC class II elements with TIL 1087 and were capable of processing other exogenous Ag such as tetanus toxoid, tyrosinase, mutated TPI, and NYT-ESO-1. These results suggested that the tumor Ag recognized by CD4+ TIL 1087 was processed through the endogenous but not the exogenous MHC class II pathway. Our findings indicated that molecular cloning would be the preferred strategy for identifying this Ag, because the alternative biochemical purification approach depends on pulsing sequentially purified protein fractions onto APC for processing through the exogenous pathway for T-cell recognition (8).

To apply a molecular cloning approach, it was necessary to determine the MHC class II restriction element for the 1087-mel Ag (4). Anti-MHC mAbs were used to inhibit recognition of 1087-mel by autologous CD4+ T cells, and recognition was abrogated not only by the mAb IVA12 specific for HLA-DR, -DP, and-DQ, but also by the mAb L243 specific only for HLA-DR molecules (8). Antibodies specific for MHC class I molecules (W6/32) or HLA-DQ molecules (Genox 3.53, IVD12; all mAbs were from ATCC hybridomas) were not inhibitory (data not shown). Genotyping revealed that 1087-mel had the potential to express the HLA-DR molecules B1*0701, B1*12, B3*02, and B4*01, and mAbs specific for each of these were not available. However, by sequencing individual cDNA clones obtained with RT-PCR using one set of primers capable of amplifying all of these HLA-DR molecules from 1087-mel cells, we detected the presence of only DRB1*0701 and DRB4*01, and DRB1*0701 clones predominated. Flow cytometric analysis with a HLA-DR7-specific mAb confirmed expression of this MHC molecule on the surface of 1087-mel cells. Thus, DRB1*0701 was selected as the restriction element for the initial cDNA library screening, and this molecule was transfected stably into 293 cells already engineered to express other components of the class II processing pathway: full-length β2m, Ia, DMB, and ERA (4). The resulting 293MIMDR7 cells were used as host cells for transient transfection of a cDNA library prepared from 1087-mel cells and ligated into the expression vector pBl80. This vector was designed for translation of protein products fused at the NH2-terminus to the first 80 amino acids of β2m, containing a endosomal targeting sequence for efficient processing of class II-restricted epitopes (4, 5, 10).

A library of 1 × 10^5 cDNA clones from 1087-mel cells was screened in pools of 100 clones for CD4+ T-cell recognition, and a single pool was identified that repeatedly stimulated cytokine secretion from TILs. After subcloning, a 1.8-kb cDNA clone, designated IB11, was isolated that conferred T-cell recognition on transfection into 293IMDR7 cells. DNA sequencing and database searching revealed that the IB11 cDNA sequence did not bear significant similarity to any sequence encoding a human protein of known function. However, it shared 97–100% identity with sequences in the human expressed sequence tag (EST) and genome project databases derived from melanoma as well as from fetus, placenta, and a wide range of cancers arising from brain, lung, stomach, endometrium, prostate, and other sites, which suggested that the encoded protein might be widely expressed in human malignancies. In cDNA IB11, the longest open reading frame of 0.5 kb was not preceded by an initiation codon but was in-frame with the li 1–80 fusion tag and was, thus, predicted to encode a 167-amino acid COOH-terminal protein fragment. To determine whether the recognized epitope derived from 1087-mel cells was mutated, sequence-specific oligonucleotide primers were used for RT-PCR amplification of the same partial cDNA fragment from autologous EBV-transformed B cells, for ligation into the pBl80 vector. Surprisingly, although TIL 1087 recognize whole autologous

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melanoma cells but not whole EBV-B cells, the partial cDNAs derived from both 1087-mel and 1087-EBV cells had identical sequences, and both of them encoded proteins that were recognized by CD4+ TIL 1087 after transient transfection into 293IMDR7 cells (Fig. 1). Notably, whereas a 1087-EBV-derived clone ligated in-frame with the Ii fusion sequence was well recognized by T cells (pIi80/IB11), an out-of-frame clone (EBV.2) was not recognized. As a control, none of these constructs, when transfected into 293IMDR1 cells, was recognized by DR1-restricted CD4+ TIL 1558, which instead were specific for mutated TPI (3). In addition, the reactivity of CD4+ TIL 1087 against 293IMDR7 transfecants was inhibited by the anti-HLA-DR mAb L243, and TIL1087 failed to recognize cDNA IB11 when transfected into MHC-incompatible 293IMDR1 cells (not shown). Taken together, these results suggest that CD4+ TIL 1087 recognize an HLA-DR7-restricted nonmutated epitope encoded by cDNA clone IB11, and that epitope recognition depends on endosomal targeting.

To identify the HLA-DR7-restricted peptide recognized by TIL 1087, 15-mer peptides overlapping by 10 amino acids were synthesized spanning the putative 167-amino-acid sequence encoded by cDNA IB11. These were tested for their ability to stimulate TIL 1087 after being pulsed onto autologous B cells. One stimulatory peptide was identified at the extreme COOH terminus of the molecule. As shown in Fig. 2A, a series of shorter overlapping peptides based on this sequence were then synthesized and tested for T-cell recognition, identifying a minimal 11-mer epitope with the sequence RVIKNSRLTLT (Fig. 2B). The identity of this peptide sequence was confirmed as a stimulatory epitope by transient transfection into 293 cells, and the peptide was shown to stimulate cytokine secretion from T cells. Gentically modified 293 cells (293IMDR7 for TIL 1087, 293IMDR1 for TIL 1558) were transfected with the indicated plasmids, as described in “Materials and Methods,” and then were cocultured with CD4+ T cells. TIL 1087 secreted GM-CSF specifically in response to autologous whole 1087-mel cells but not 1087-EBV cells. We hypothesized that a distant mutation upstream from the recognized epitope might indirectly influence processing events, similar to results observed with the CDC27 Ag (4). Therefore, 5′ rapid amplification of cDNA ends (RACE) was used with sequence-specific primers as previously described to obtain the complete coding DNA sequence of the recognized molecule (6). This led to the identification of a 3.7-kb cDNA with a 2.2-kb open reading frame, predicted to encode a protein of 736 amino acids. This protein sequence, although not identical to any sequence in the published databases, had an overall similarity of 71% to human PAP, an RNA-processing enzyme that catalyzes the addition of the 3′ poly(A) tail to pre-mRNA (6). Thus the new protein was designated neo-PAP (GenBank accession no. AF312211). The organization of neo-PAP seemed to recapitulate that of classic PAP, including an NH2-terminal catalytic domain and two COOH-terminal nuclear localization sequences flanking a putative regulatory domain that contained multiple phosphorylation sites. Although similar in sequence to nonhuman vertebrate PAPs as well, the neo-PAP protein was not significantly similar to any other human proteins with known functions.

Fig. 1. Specific recognition of cDNA IB11 by CD4+ TIL 1087. Genetically modified 293 cells (293IMDR7 for TIL 1087, 293IMDR1 for TIL 1558) were transfected with the indicated plasmids, as described in “Materials and Methods,” and then were cocultured with CD4+ T cells. TIL 1087 secreted GM-CSF specifically in response to autologous whole 1087-mel cells and to 293 cells transfected with pIi80/IB11 mel or pIi80/IB11 EBV.1, but not with the out-of-frame construct pIi80/IB11 EBV.2 nor the irrelevant plasmid pIi80/TPImut. Conversely, TIL 1558 recognized autologous melanoma cells and 293 cells transfected with the mutated TPI construct but were not stimulated by IB11 transfectants.
as described previously (6). DNA sequencing revealed a C/H11022T point mutation that occurred in 5 of 15 melanoma clones, but none of 7 B cell clones sequenced. C/H11022T nucleotide substitutions, frequently observed in melanomas, have been linked to UV irradiation damage (summarized in Ref. 3). As shown in Fig. 3A, this mutation in neo-PAP was predicted to cause a nonconservative P/H11001L amino acid substitution at residue 643 situated in a putative phosphorylation site, immediately preceding the second nuclear localization sequence 80 amino acids upstream from the recognized nonmutated epitope. The disruption of a phosphorylation site or of a nuclear localization motif could impact on protein stability and/or trafficking, which suggests mechanisms by which Ag processing might be influenced. In addition, proline is unique among amino acids in that its α-nitrogen is part of a rigid cyclic ring, and, thus, its presence in any peptide or protein has distinct conformational effects that can have important consequences for the overall structure, function, and stability of the molecule (11, 12). Furthermore, prolines generally confer protection from proteolytic cleavage and, as a result, have been shown to inhibit certain processing events in the MHC class I pathway (13). Thus, there are several potential mechanisms by which a mutation of P643 could influence the processing of neo-PAP. Conveniently, the C>H11022T nucleotide mutation in neo-PAP also created a new BfrI enzymatic cleavage site (ctag>cctagg), allowing for the rapid screening of other tissues for its presence. As shown in Fig. 3B, RT-PCR products of 0.9 kb encoding the COOH terminus of neo-PAP (6) were partially cleaved by BfrI into fragments of 0.3 + 0.6 kb if derived from the fresh cryopreserved 1087 melanoma tumor or from the cultured 1087-mel cell line. No cleavage was observed in DNA amplified from an allogeneic melanoma line, 888-mel. These results demonstrate that the 1087 tumor expresses mRNA encoding both the wild-type (uncut) and mutant alleles of neo-PAP, and that the presence of this mutation in the 1087-mel cell line that was used to make the cDNA library is not an artifact of in vitro culture. In addition, this mutation was not found in 1087-EBV B cells nor in fresh 1087 peripheral blood lymphocytes, which indicates that it is not likely to represent a polymorphism. As detailed in Ref. 6, studies of a total of 21 tissues and cell lines failed to detect this point mutation in any source except the 1087 melanoma.

Subcellular Localization of neo-PAP. In an effort to determine the mechanism by which the mutation of neo-PAP found in 1087-mel cells could lead to processing of the nonmutated immunogenic epitope, we used immunofluorescence microscopy to examine the subcellular localization of both the wild-type and mutant proteins. Lacking a mAb specific for neo-PAP, we engineered epitope tags detectable by specific mAb onto wild-type and mutant neo-PAPs for this purpose. We have previously demonstrated that wild-type neo-PAP localizes exclusively to the nucleus in HeLa cells transiently transfected with an NH2-terminal HA epitope-tagged molecule (6). Because the mutation associated with TIL recognition has the poten-

![Image](https://example.com/image.png)

**Fig. 3.** Demonstration of a mutant neo-PAP allele in 1087-mel. A, amino acid sequences of the wild-type and mutant alleles of neo-PAP. Asterisks, a nuclear localization sequence; bolded residues, putative phosphorylation sites; underlined, the epitope recognized by CD4+ T cells. B, restriction digestion of RT-PCR products demonstrates the presence of a mutated allele in neo-PAP derived from fresh and cultured 1087 melanomas. As a negative control, neo-PAP from 888-mel is not cleaved. BfrI cleavage products were electrophoresed on a 1% agarose gel. These results were confirmed by DNA sequencing.

![Image](https://example.com/image.png)

**Fig. 4.** Subcellular localization of wild-type and mutant neo-PAPs. After a 20-h transfection, HeLa cells were stained with mAbs specific for the NH2-terminal HA epitope (green) and the COOH-terminal FLAG epitope (red) fused to neo-PAP. a–c, wild-type neo-PAP; d–f, mutant neo-PAP. For both neo-PAPs, the NH2 and COOH termini colocalize to the nucleus (yellow). ×980.
tial to result in altered trafficking or cleavage of neo-PAP, a FLAG tag was added to the COOH terminus juxtaposed to the recognized 11-mer peptide, theoretically allowing discrimination of stable cleavage products trafficking to different intracellular compartments with two-colored staining. Fig. 4 shows that, after a 20-h transfection in HeLa cells, both the wild-type and the mutant neo-PAP molecules seemed to localize to the cell nucleus, with complete colocalization of the NH$_2$ termini stained by an anti-HA mAb (green) and the COOH termini stained by an anti-FLAG mAb (red). No significant staining of the cytoplasm was observed. Thus, both molecules appeared to localize similarly during the limited period of observation. In addition, Western blotting on lysates of transiently transfected 293 cells failed to demonstrate cleavage products from the mutant or wild-type neo-PAPs, whether staining was done with the anti-HA or the anti-FLAG mAb (data not shown). It is unknown how the engineered epitope tags might have influenced the behavior of the wild-type and mutant molecules, and efforts are currently underway to develop a mAb specific for neo-PAP to allow its visualization in 1087-mel cells.

The revelation of a cryptic MHC class II-restricted epitope in neo-PAP by a unique tumor-associated mutation provides insight into the kinds of genetic aberrations that can unveil otherwise silent transcripts for immune recognition. A number of normally invisible epitopes have been described as emanating from alternative reading frames, antisense transcripts, pseudogenes, and chromosomal translocation (reviewed in Ref. 14). Although the exact mechanism by which mutant neo-PAP is processed for immune recognition remains to be solved, important clues are provided by the critical location and nonconservative nature of the tumor-associated mutation. The mutant variant of neo-PAP identified in patient 1087 appears to be unique and, therefore, not generally applicable in an immunotherapeutic context. However, future investigation will address whether the wild-type neo-PAP Ag, overexpressed in a variety of human cancer types, can be recognized by other cancer patients and by other arms of the antitumor immune response. In addition, the discovery of neo-PAP, a novel RNA processing enzyme the function of which appears to be regulated differently from that of the homologous classic PAP, has provided a basis for ongoing studies of mRNA processing events and their potential aberrations in malignant cells.

ACKNOWLEDGMENTS

We gratefully acknowledge Samuel Johnston for cloning HLA-DR7, John Riley and Maria Parkhurst for peptide synthesis, and Yong Li and Aaron Chen for DNA sequencing. We thank Paul Robbins and Jim Manley for helpful discussions. We also thank Drew Pardoll for ideas and critical review of the manuscript, and Steven A. Rosenberg for advice and support.

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