A Processed Pseudogene Codes for a New Antigen Recognized by a CD8 T Cell Clone on Melanoma

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Abstract

The M88.7 T cell clone recognizes an antigen presented by HLA B*1302 on the melanoma cell line M88. A cDNA encoding this antigen (NA88-A) was isolated using a library transfection approach. Analysis of the genomic gene’s sequence identified it is a processed pseudogene, derived from a retrotrotranscript of mRNA coding for homeoprotein HPX42B. The NA88-A gene exhibits several premature stop codons, deletions, and insertions relative to the HPX42B gene. In NA88-A RNA, a short open reading frame codes for the peptide MTQGQHFLQKV from which antigenic peptides are derived; a stop codon follows the peptide’s COOH-terminal Val codon. Part of the HPX42B mRNA’s 3′ untranslated region codes for a peptide of similar sequence (MTQGQHFSQKV). If produced, this peptide can be recognized by M88.7 T cells. However, in HPX42B mRNA, the peptide’s COOH-terminal Val codon is followed by a Trp codon. As a result, expression of HPX42B mRNA does not lead to antigen production. A model is proposed for events that participated in creation of a gene coding for a melanoma antigen from a pseudogene.

Key words: peptide • epitope • processing • tumor immunity • CTL

Introduction

CTLs can recognize antigens presented by human tumor cells. In the hope that manipulating this CTL response to combat cancer will prove possible, a number of groups have started to identify the antigens in question (for review see reference 1). The results of several clinical trials of both adoptive immunotherapy (2) and vaccination (3–5) based on this work suggest that the effort is worthwhile.

Most of the antigens characterized to date are HLA class I restricted and presented by melanoma cells. Many of them are derived from normal or mutated gene products obtained by classical pathways of gene expression (1). However, an increasing number of reports describe antigens resulting from the use of nonclassical pathways of gene expression, when unexpected events take place during transcription, splicing, or translation (6). Thus, the NA17-A antigen (7) is translated from a transcript obtained by activation of a cryptic promoter within intron sequences of the N-acetylglucosaminyltransferase-V gene. Reverse strand transcription of a housekeeping gene gives rise to an antigen recognized by CTLs on a human kidney tumor (8). Intron retention during splicing is responsible for generation of some antigens from the gp100 (9) and tyrosinase-related protein (TRP)-2 (10) genes. Use of alternative open reading frames (ORFs) for translation can also create antigens, as described for a breast and melanoma-shared antigen (11), a gp75TRP-1 antigen (12), a renal cell carcinoma antigen derived from the intestinal carboxyl esterase gene (13), and an antigen derived from the LAGE-1 gene (14).

We describe here a new tumor antigen generating an HLA class I–restricted CTL response against melanoma. Surprisingly, the antigen is coded for by a processed pseudogene. Expression of the “living” counterpart of the pseudogene does not lead to a corresponding CTL response. This difference between the two genes provides insights into how a gene coding for a tumor antigen was created.


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Materials and Methods

Cell Lines. Melanoma cell lines (M) were established from metastatic tumor fragments from different patients. IPC 277/5 and IGR 1/54 melanoma cell lines were gifts from C. Aubert (INSERM U119, Marseille, France). The FM2.29 cell line was a gift from J. Zeuthen (Danish Cancer Society Research Center,
Copenhagen, Denmark). D A U V melanoma cell line and mouse fibrosarcoma W E H I 164 clone 13 used for T N F assays was obtained from T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). T cell clone M 88.7 was derived from tumor infiltrating lymphocytes of patient M 88 (A'0201, A'0301, B'4002, B'1302, Cw'02022, Cw'0602) as described previously (15).

H L A T ransfection in T umor C e l l L ines. C ells were seeded in 96-well plates and incubated until 80% confluent before addition of expression vector (100 ng) and Lipofectamine (0.5 μl) in serum-free medium. After 12–15 h, the transfection mixture was replaced with complete medium. Transfected tumor cell lines were used for stimulation of T cell clone M 88.7 36–48 h after transfection.

Library Screening. Poly A+ R N A was obtained from M 88 cells using the FastTrack 2.0 m RN A extraction kit (Invitrogen Corp.). m RN A was converted to cDNA using a cDNA synthesis kit (Stratagene Inc.). The cDNA obtained was inserted into pCl-neo (Promega). R ecoombinant plasmids were electroplated into E scherichia coli X L 2–2 Blue M R F (Stratagene Inc.). For screening, 1,500 pools of 100 ampicillin-resistant bacteria per pool were constituted. Plasmid D N A was extracted by the alkaline lysis method from each pool (16). For genomic gene cloning, a h uman cosmid library (provided by A. Hauanuer, J G B M C, Strasbourg, France) was hybridized with the 2.7-kb N A 88-A cloned cDNA. Selected regions of the positive cosmid identified were sequenced using the Applied Biosystems 373 D NA sequencer (Perkin Elmer).


transfection of C O S -7 C ells. D E A E - dextran-chloroquine transfection was as described (17). In brief, 1.5 × 10^4 COS-7 cells were transfected with 100 ng of the H LA B*1302 expression vector and 100 ng of a pool of the cDNA library. Transfectants were tested after 48 h for their ability to stimulate T N F production by T cells. One positive pool was identified, and 500 individual plasmids obtained from it tested for the ability to trigger T cell T N F release as described for pools.

T C l e l Stimulation A ssay. T cells (2.5 × 10^3 per well) were added to COS-7 cells or tumor cells 24–48 h after transfection. Culture supernatants were harvested 6 h later and tested for T N F content by measuring culture supernatant cytotoxicity to W E H I 164 clone 13 in a colorimetric assay (18).

Production of M odified D N A s. T unced N A 88-A cDN As were generated using standard techniques (16) and cloned into pCDNA3 (Invitrogen Corp.) or into pCl-neo. pNA88-A Trp, pNA88-A Ser, and pH PeX2 Stop were generated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene Inc.).

Reverse Tr anscrip tase-P C R A ssay s. T otal R N A was extracted by the guanidinium–cesium chloride procedure (16) and used for cDNA syn thesis (19). Some samples of tumor and normal tissue cDNA were provided by F. Brasseur (Ludwig Cancer Institute, Brussels, Belgium). To test for genomic DNA contamination, cDNA samples were used for PCR with primers designed to amplify the M A G E -1 genomic gene. One of the samples used here gave a positive result in this test. For amplification of N A 88-A cDNA, PCR assays were performed with primers 5'-CCT-TGTGGTTGATGCCC-3' and 5'-GGACACGGCGCACAGGACC-3' for 35 cycles (1 min at 94°C, 1 min at 62°C, and 1 min 30 s at 72°C). Under these conditions, primers will not amplify H P X 428 cDNA. PCR products were size fractionated and blotted to Hybond-N+ (Amersham Pharmacia Biotech) before hybridization with the cloned N A 88-A cDNA. Samples were normalized for R N A and cDNA integrity and quantity by PCR amplification of human β-actin cDNA (19).
ety of normal and tumor tissues. We were unable to detect NA88-A RNA in any of the normal tissues tested, including normal melanocytes in culture, with the possible exception of the testis (only two samples were tested, though both proved positive; data not shown). Among the tumor samples we tested, expression of NA88-A RNA was limited to some metastatic melanomas (7 of the 66 tested; data not shown).

NA88-A RNA Is Similar to the Homeoprotein-Encoding HPX42B mRNA. There was no long ORF on the 2.7-kb NA88-A cDNA, although it did contain an AATAAA polyadenylation signal and terminal poly A tail. While the...
sequence was new, its first 1,048 nucleotides proved to be ~90% identical to two noncontiguous parts (separated by an Alu family member) of the 3' untranslated region (UTR) of the previously reported HPX42B cDNA (Fig. 2A; similar sequences are shown in black). HPX42B cDNA was originally obtained in a search for new homeobox genes, using degenerate PCR on cDNA derived from a purified population of CD34+ human hemopoietic cells (20). The similarity between the NA88-A and HPX42B sequences suggested that the cloned NA88-A cDNA could be part of the 3' UTR of a homeoprotein-encoding gene related to HPX42B. To test this hypothesis, we decided to isolate the genomic NA88-A gene by screening a human cosm id library.

A Processed Pseudogene Encodes NA88-A RNA. We isolated one NA88-A cosm id and sequenced part of it to search for sequences coding for a HPX42B-like protein. The NA88-A sequence determined is shown in Fig. 2B, aligned with the corresponding HPX42B gene sequence.

(A region of chromosome 10 including the HPX42B has been sequenced. Comparison of this sequence with the HPX42B cDNA shows that the gene contains two introns. Their positions, but not their sequences, are marked in Fig. 2B.) The NA88-A and HPX42B sequences are very similar. However, relative to the HPX42B gene, the NA88-A gene contains a number of mutations. One point mutation converts codon 22 of the HPX42B gene (tgg) into a premature stop codon (tga; boxed in Fig. 2B). Relative to the HPX42B gene, the NA88-A gene also contains a number of deletions and insertions. While several of these remove or add codons without changing the reading frame, two of the deletions (of 1 and 80 bp) and one insertion (of 2 bp) do change it. As a result of all these mutations, the NA88-A gene no longer codes for a homeoprotein.

Further comparison of the genes shows that the NA88-A gene lacks equivalents of the HPX42B gene's introns (Fig. 2, B and C). These observations strongly suggest that the NA88-A gene is a processed pseudogene, resulting from

Figure 3. Identification of the antigenic peptide. (A) Translation products of nucleotides 674–730 of NA88-A cDNA in all three reading frames. 1, 2, and 3: peptides synthesized. (B) Production of TNF by M88.7 T cells in response to M88 cells or one of the three synthetic peptides shown in A as marked. (C and D) Determination of the minimal antigenic peptide. Production of TNF (C) and IL-2 (D) by the M88.7 T clone in response to different synthetic peptides derived from peptide 1. For A, T cell clone M88.7 was stimulated with different concentrations of each peptide. For B, T cell clone M88.7 was stimulated with 50 μM peptide. Fixed, stimulated cells were stained for cytokine and analyzed on a FACScan™. Percentages of positive cells are indicated in the dot plots.
chromosomal integration of part of a retrotranscript of HPX42B mRNA. For splicing of the first HPX42B intron, the HPX42B mRNA employed to generate the NA88-A gene appears to have used an alternative 3' splice site (3'5S in Fig. 2, B and C). We cannot tell how the second intron was removed, as the NA88-A gene contains a large deletion covering the region corresponding to the junction between HPX42B exons 2 and 3. One hallmark of processed pseudogenes is the presence of an A-rich sequence corresponding to the poly A tail of the original mRNA. However, as the NA88-A gene lacks sequences corresponding to the end of the HPX42B mRNA's 3' UTR (Fig. 2A), this A-rich sequence is not expected to be present in the NA88-A pseudogene. In conclusion, our data show that the NA88-A antigen is coded for (a) by a processed pseudogene and (b) by sequences derived from the 3' UTR of the HPX42B mRNA.

Identification of the Peptide Recognized by M88.7 T Cells. To identify the region of the 2,729-bp NA88-A cDNA coding for the antigenic peptide, we tested a number of subfragments for their ability to direct antigen production. TNF was produced by M88.7 T cells exposed to COS-7 cells cotransfected with pHLA B*1302 and vectors containing the NA88-A cDNA region 1–722, but not by M88.7 T cells exposed to COS-7 cells cotransfected with pHLA B*1302 and vectors containing the NA88-A cDNA region 1–700 (Fig. 3A). These results positioned the end of the sequence coding for the antigenic peptide between nucleotides 700 and 722. Peptides covering all three reading frames encoded by the cDNA region 684–730 were synthesized (Fig. 3A) and tested for their capacity to induce TNF production by M88.7 T cells. Only one of them led to significant TNF production (peptide 1, RMTQGQHF-LQKV; Fig. 3B).

To characterize the antigenic peptide further, two 10-mers and two 9-mers representing different but overlapping regions of the 12-mer peptide 1 were tested in parallel for their capacity to induce secretion of TNF by M88.7 T cells (Fig. 3C). Results obtained show (a) that removal of the COOH-terminal valine abolishes peptide activity and (b) that the 10-mer (TQGQHFLQKV) and the 9-mer (QGQHFLQKV) with this valine present are both as active as the parent 12-mer. A similar conclusion was reached when IL-2 production by M88.7 T cells exposed to the peptides was measured (Fig. 3D).

HPX42B mRNA Expression Does Not Lead to T Cell Stimulation. An alignment of the HPX42B and NA88-A cDNA sequences covering the region of the NA88-A sequence coding for the antigenic peptide is shown in Fig. 4A. Part of the 3' UTR of the HPX42B cDNA can potentially encode the peptide MTQGQHSFKV, which differs from the NA88-A antigenic peptide by a single substitution, serine (underlined) for leucine. To test if HPX42B mRNA expression leads to stimulation of M88.7 T cells, COS-7 cells were cotransfected with pHLAB*1302 and HPX42B (Fig. 4B), an expression vector containing the entire HPX42B cDNA. Transfected cells were unable to
stimulate TNF production by M 88.7 T cells (Fig. 4 C). However, COS-7 cells cotransfected with pH LAB*1302 and a point-mutated NA 88-A expression vector coding for the serine-containing HPX4 2B peptide (pNA 88-A Ser; Fig. 4 B) did stimulate TNF production by M 88.7 T cells (Fig. 4 C), albeit less well than cells transfected with wild-type pNA 88-A. Thus, although both NA 88-A RNA and HPX4 2B mRN A code for a peptide capable of stimulating M 88.7 T cells, only NA 88-A RNA expression leads to peptide presentation.

A stop codon in the NA 88-A gene is vital for the T cell response. In NA 88-A RNA, the codon coding for the COOH-terminal Val of the antigenic peptide is followed by a stop codon, whereas in HPX4 2B mRNA, the corresponding Val codon is followed by a Trp codon (Fig. 4 A). This difference is important. COS-7 cells cotransfected with pH LAB*1302 and pH 88-A Trp (Fig. 4 B), in which the stop codon has been replaced by the Trp codon found in HPX4 2B mRNA, were unable to stimulate significant TNF production by M 88.7 T cells (Fig. 4 C). However, COS-7 cells cotransfected with pH LAB*1302 and pH 4X2B Stop (Fig. 4 B), in which the Trp codon has been mutated to a stop codon, were able to stimulate TNF production by M 88.7 T cells (Fig. 4 C). TNF levels obtained were similar to those obtained using pH 88-A Ser, which codes for the same serine-containing peptide.

**Discussion**

The NA 88-A gene is a processed pseudogene, which no longer codes for any likely functional protein. The NA 88-A antigen is encoded by a very short ORF, which was derived from part of a functional mRNA’s 3’ UTR. Our results thus extend the possible sources of tumor antigen coding sequences to “junk” DNA and seriously raise the possibility that any DNA sequence can lead to antigen production, the only limitation being that it must be transcribed. As is not surprising for junk DNA, transcription of the NA 88-A gene is limited. Significant expression is found only in a fraction of metastatic melanomas. Transcription of the gene may occur only at a late stage in melanoma, after multiple genetic changes have accumulated facilitating aberrant transcription.

In contrast, the NA 88-A’s “parent” gene, the HPX4 2B gene, is transcribed in a variety of normal tissues (our unpublished observations). The 3’ UTR of HPX4 2B mRNA has codons for a peptide that can be recognized by M 88.7 T cells. However, unlike for NA 88-A RNA, expression of HPX4 2B mRNA does not lead to T cell stimulation. One reason is that antigenic peptides must be excised from a precursor of 125 amino acids (which begins MTQGQHF-SQKFWKCP EWE...; antigenic peptide in bold). Thus, expression of a mutated HPX4 2B mRNA, in which the Trp codon immediately following the peptide’s terminal Val codon, is replaced by a stop codon, will lead to T cell stimulation. The absence of M 88.7 T cell stimulation after wild-type HPX4 2B mRNA expression can be explained if downstream flanking residues stop appropriate peptides from being produced in quantities sufficient for presentation. Proteolytic cleavage COOH-terminal to the peptide’s Val residue may be inefficient. Other cleavages may be favored that lead to peptides containing MTQGQHF SQK KV but that cannot be presented productively to M 88.7 T cells, due to a problem with peptide stability, TAP (transporter associated with antigen processing) transport, or MHC loading. Alternatively, the downstream residues could provoke cleavage within the MTQGQHF SQKV sequence itself. In favor of these suggestions, a number of reports exist (21–28) describing the influence of flanking residues on epitope production and/or presentation.

None of the above problems is likely to adversely affect production of NA 88-A antigenic peptide. The peptide MTQGQHFLOKV is the direct translation product of a short ORF (a stop codon follows the Val codon), and antigenic peptides can be derived from it after at most minimal NH2 terminal trimming. In addition to avoiding potential problems with precursor processing (see above), the lack of any significant processing requirement should limit the level of transcription and translation required to produce sufficient levels of peptide for T cell stimulation. This could be important, as the NA 88-A RNA contains no primary ORFs, but rather many short ORFs, and there is no obvious reason why the ORF coding for the antigenic peptide should be chosen for translation rather than any of the others. Translation of the antigenic peptide’s ORF is thus likely to be inefficient.

In conclusion, the NA 88-A gene codes for a melanoma-specific antigen today, in large part because, during its evolution, a point mutation transformed a Trp codon into a stop codon. This significantly augmented production of antigenic peptide.

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