A Single-nucleotide Deletion Leads to Rapid Degradation of TAP-1 mRNA in a Melanoma Cell Line*

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Both viruses and tumors evade cytotoxic T lymphocyte-mediated host immunity by down-regulation of antigen presentation machineries. This can be achieved by either down-regulation of transcription of antigen presentation genes or posttranslational inactivation of proteins involved in antigen presentation. In this study, a major histocompatibility complex (MHC) class I-deficient melanoma cell line, SK-MEL-19, was found deficient in the expression of the transporter associated with antigen processing (TAP) mRNA even after IFN-γ stimulation, despite its active transcription of the TAP-1 gene. This abnormality was caused by a single-nucleotide deletion at position +1489 of the TAP-1 gene and was corrected by cycloheximide, which inhibits RNA degradation. Using an inducible Tet-Off system, we demonstrated that deletion of the nucleotide resulted in a >2-fold decrease in the half-life of TAP-1 mRNA. However, the decrease of the half-life of TAP-1 mRNA is not mediated by nonsense-mediated mRNA decay because deletions of two additional nucleotides in the region, which corrected the nonsense mutation, did not restore TAP-1 mRNA stability. To our knowledge, this is the first evidence that the degradation of mRNA of an antigen presentation gene is involved in HLA class I down-regulation in malignant cells.

Recent studies demonstrate that patients with malignant melanoma often have a high number of cytotoxic T lymphocytes specific for melanoma-associated antigens (1, 2). The co-existence of T cells and tumor cells even in the draining lymph nodes suggests that the tumors were able to evade destruction by host cytolytic T lymphocytes. Accumulating evidence supports the notion that both malfunction of T cells and down-regulation of antigen presentation machinery in tumors can be responsible for tumor evasion of host immunity (1–6). In fact, a high proportion of malignant tumors, including melanoma, have severely depressed cell surface expression of class I HLA antigens (7), the target molecules that present tumor antigenic peptide to cytolytic T lymphocytes. Understanding the mechanisms underlying the T-cell malfunction or antigen presentation defects may thus provide insight for immunotherapy of melanoma and other cancers.

Optimal cell surface expression of HLA molecules requires the coordinated expression of several genes, such as transporters associated with antigen processing (TAP)γ-1/2, low molecular weight peptide (LMP)-2/7, and tapasin, as well as HLA class I heavy chain and β2-microglobulin (β2-M). In cases of both tumorigenesis and viral infection, expression of these genes and the function of the encoded proteins are often impaired (8–10). The mechanisms for such down-regulation have been studied extensively. Theoretically, gene expression can be modulated by transcriptional, posttranscriptional, translational, and posttranslational mechanisms. The mechanisms that have been shown to underlie the antigen presentation abnormalities are transcriptional suppression of antigen presentation genes and/or functional inactivation of their gene products, either by missense mutation or by protein-protein interactions (11–14). Here we show that actively transcribed TAP-1 mRNA in the melanoma cell line SK-MEL-19 is rapidly degraded even after stimulation with IFN-γ. Cloning and sequencing analysis have revealed a single-nucleotide deletion at position +1489. This mutation results in substantial reduction of the stability of TAP-1 mRNA by mechanisms unrelated to nonsense-mediated mRNA decay (NMD). These results reveal a new potential mechanism for tumor evasion of host T-cell recognition.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—Human melanoma cell lines 1195, 1102, and SK-MEL-19 were cultured as described previously (15). The breast cancer cell line SK-BR-3 was obtained from ATCC (HTB-30; ATCC, Manassas, VA). All cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mm l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). For induction of HLA class I expression, cells were cultured in medium supplemented with recombinant human IFN-γ (1000 units/ml; R&D Systems, Minneapolis, MN). PE-conjugated anti-HLA-A, -B, and -C antibody (clone G46-2.6) and isotype control PE-conjugated mouse IgG1 were purchased from BD PharMingen (San Diego, CA).

Flow Cytometry—Cell surface HLA class I expression was examined by flow cytometry as described previously (4). Briefly, viable cells were incubated with PE-conjugated mouse IgG1 and PE-conjugated anti-HLA-A, -B, and -C antibody at 4 °C for 2 h. After three washes with phosphate-buffered saline containing 1% fetal calf serum, cells were fixed with 1% paraformaldehyde and examined by flow cytometry.

Northern Blot—Cells were either treated with IFN-γ (R&D Systems) at 1000 units/ml for 48 h or left untreated. For cycloheximide (CHX; Sigma) treatment, SK-MEL-19 cells were cultured with IFN-γ at 1000 units/ml for 48 h, and then CHX was added to the cells for a final concentration of either 5 or 10 μg/ml, respectively, for up to 16 h. Total RNA was isolated using TRIzol reagent (Invitrogen). Hybridization conditions followed the instructions of the Northern hybridization kit.
mRNA Instability and TAP-1 Down-regulation in Tumor

The human small cell carcinoma H146 cell line (provided by Dr. N. P. Restifo, National Cancer Institute, Bethesda, MD) was incubated with IFN-γ at 1000 units/ml for 48 h. Total RNA was isolated as described above. The cDNA was synthesized using the SuperScript II Reverse Transcriptase Kit (Invitrogen) and reamplified in one step using the POWER SYBR Green PCR kit (Applied Biosystems). The PCR products were separated by agarose gel electrophoresis and purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA).

Generation of TAP-1 cDNA Constructs and Stable Transfection—The small cell carcinoma H146 cell line was due to the TAP-1 down-regulation detected by Northern blot. The mRNA expression was inhibited by at least 95% by tetracycline treatment. The cell clones in which TAP-1 mRNA expression was inhibited by at least 95% by tetracycline treatment were used for the study of mRNA stability.

RNAse Protection Assay—The TAP-1 cDNA construct used to make the TAP-1 antisense transcript was the same as the one used for the Northern blot. The GAPDH cDNA fragment was generated by PCR

The total cDNA from the SK-MEL-19 cells was used as the template for each PCR. After confirming their sequences, all three TAP-1 cDNAs were inserted into the multiple cloning site of the pBI-EGFP vector (Clontech) by blunt-end ligation. The three constructs, pBIEGF-TAP1, pTet-Off (Clontech), and pcDNA3.1/Hyg (+) (Invitrogen), were co-transfected into SK-MEL-19 cells using FuGENE 6 transfection reagent (Roche Molecular Biochemicals). Stably transfected cell clones were selected in 96-well plates in Dulbecco's modified Eagle's medium supplemented with 0.5 mg/ml hygromycin B (Invitrogen). Single cell clones were selected for further culture and analyzed for HLA class I antigen expression.

Southern Blot—Genomic DNA was isolated from SK-MEL-19 cells, SK-BR-3 cells, and HeLa cells. Genomic DNA (20 μg) was digested with AIII (Invitrogen) and separated in 0.8% agarose gel. The TAP-1 promoter probe was made by PCR from normal human lymphocyte genomic DNA with sense primer 5'-TACAGGGAGTGGTAGGTTGTACCTG-3' and antisense primer 5'-GGCAAGCCTGTTGCTATCGG-3' (Promega, Madison, WI) at XhoI and HindIII sites. All constructs were confirmed by DNA sequencing. Expression level of the firefly luciferase from the pGL2 constructs (Promega) was normalized to the internal control of HLA class I heavy chain (MHCI), pβ-M, TAP-1, TAP-2, and LMP-7 in each cell line with or without IFN-γ induction (1000 units/ml for 72 h). Total RNA loading to each well was shown as 28 S rRNA (28S) and 18 S rRNA (18S) cDNA inserts insert (bottom panels). These stable clones from each group were stimulated with or without IFN-γ and analyzed for cell surface HLA-A, -B, and -C, as detailed in a previous report.

FIG. 1. Deficiency of surface HLA class I expression in melanoma cell line SK-MEL-19 was due to the TAP-1 down-regulation. a, HLA class I expression in three melanoma cell lines, SK-MEL-19, 1102, and 1196. Bold black lines depict the staining by PE-conjugated anti-human HLA-A, -B, and -C antibody in untreated cells, dotted lines represent the staining by PE-conjugated mouse IgG1 as isotype control, and red lines represent anti-HLA-A, -B, and -C antibody staining after stimulation with 1000 units/ml IFN-γ for 72 h. b, Expression of HLA class I heavy chain (MHCI), pβ-M, TAP-1, TAP-2, LMP-2, and LMP-7 in each cell line with or without IFN-γ induction (1000 units/ml for 72 h). Total RNA loading to each well was shown as 28 S rRNA (28S) and 18 S rRNA (18S). c, transfection with wild type TAP-1, but not vector alone, restored HLA class I expression in the SK-MEL-19 cells. SK-MEL-19 cells were transfected with either vector alone (top panels) or vector with TAP-1 cDNA insert (bottom panels). These stable clones from each group were stimulated with or without IFN-γ and analysed for cell surface HLA-A, -B, and -C, as detailed in a.
Fig. 2. Posttranscriptional mechanisms are responsible for poor accumulation of TAP-1 mRNA. a, a single-nucleotide polymorphism, adjacent to the first transcription start site (−427), was identified at −446 in the bidirectional promoter shared by the TAP-1 and LMP-2 genes. The G → T change results in the loss of the A/III restriction site. Southern blot hybridization was performed using A/III and detected by a DNA probe that encompasses the downstream region of the polymorphism site. SK-MEL-19 cells showed one 5.6-kb band that represents homozygous T allele, as did the breast cancer cell line SK-BR-3, which has significant cell surface HLA class I surface expression (data not shown). HeLa cells, in contrast, have homozygous for the G allele. b, activities of T and G alleles of TAP-1 promoter (pTAP1/T and pTAP1/G, respectively) in SK-MEL-19 cells. The two allelic forms of TAP-1 promoter were cloned into pGL2-basic vector (basic) that did not contain any promoter or enhancer but encoded firefly luciferase. The pGL2-SV40 construct (SV40) that had both SV40 promoter and SV40 enhancer as well as the firefly luciferase reporter gene was used as a positive control. After transfection, IFN-γ was added to the cell culture at 1000 units/ml. Cells were lysed 48 h after transfection, and luciferase expression was tested using a luminometer. Data shown are representative of at least five independent experiments. c, the TAP-1 gene was actively transcribed in SK-MEL-19 cells in the presence and absence of IFN-γ in nuclear run-on assay. Endogenous GAPDH expression was used as a positive control, and the pcDNA3.1/Hyg(+) vector was used as a negative control. The run-on experiments were repeated three times with similar results.

RESULTS AND DISCUSSION

Down-regulation of TAP-1 mRNA by a Posttranscriptional Mechanism in Melanoma Cell Line SK-MEL-19—Three human melanoma cell lines (1102, 1195, and SK-MEL-19) were examined by flow cytometry for their cell surface HLA class I expression with or without IFN-γ stimulation. A PE-conjugated anti-human HLA-A, -B, and -C antibody was used to detect all HLA class I alleles, and a PE-conjugated mouse IgG1 was used as isotype control. As shown in Fig. 1a, 1102 and 1195 cells had significant HLA class I that was further up-regulated by incubation with 1000 units/ml IFN-γ for 3 days. Confirming previous studies (15), we found that SK-MEL-19 cells had no cell surface HLA. Surprisingly, whereas other melanoma cell lines up-regulated their cell surface HLA in response to IFN-γ, very little HLA class I antigen could be found on the SK-MEL-19 cells even after IFN-γ-treatment.

Because optimal cell surface HLA class I expression requires the coordinated expression of multiple genes, including TAP-1/2, LMP-2/7, and β2-M as well as HLA class I heavy chain, a Northern blot analysis was performed to detect the expression of these genes (Fig. 1b). In 1102 and 1195 cells, all six genes were expressed at low but detectable levels. IFN-γ treatment dramatically induced expression of all six genes. Interestingly, in the SK-MEL-19 cells, whereas β2-M, HLA heavy chain, LMP-2, LMP-7, and TAP-2 were present at low levels without induction, no TAP-1 mRNA was detected. After IFN-γ treatment, β2-M, HLA heavy chain, TAP-2, LMP-2, and LMP-7 were expressed at high levels, yet TAP-1 was still expressed at low levels.

It has been known that TAP-deficient cells can express HLA class I after transfection with the TAP-1 or TAP-2 gene (17–19). To test whether the lack of TAP-1 expression was responsible for the barely detectable expression of HLA class I antigen on the surface of SK-MEL-19 cells, we transected the cells with TAP-1 cDNA. As shown in Fig. 1c, the TAP-1 cDNA-transfected SK-MEL-19 cells expressed significant levels of HLA class I antigen even before IFN-γ-treatment. Moreover, the TAP-1 transfectants were as responsive to IFN-γ as the other melanoma cell lines. Based on these results, it is likely that the primary defect of antigen presentation in SK-MEL-19 cells is attributable to defects in TAP-1 expression.

Because the TAP-1 expression was low at the mRNA level, we hypothesized that the TAP-1 down-regulation was caused by defective transcription or malfunction in RNA metabolism. The TAP-1 expression is under the control of a bidirectional promoter, as characterized by Wright et al. (20). We cloned and sequenced the 593-bp TAP-1 promoter from SK-MEL-19 cells. In comparison with the published sequence (20), a single-nucleotide G → T replacement was identified at position −446 (the first ATG of the TAP-1 gene is designated as +1), which is close to the first transcription start site at −427 (20) (Fig. 2a). Because the T allele results in a loss of restriction site A/III, we did a Southern blot hybridization using A/III to confirm the mismatch. As shown in Fig. 2a, whereas the HeLa cell line using the primers hGAPDH.f (5′-TGAGAACGGAAGCTTGTGAGCTG-3′) and hGAPDH.r (5′-GCCCTTCTAGATGCTGATTGC-3′). The antisense probes were made using the Riboprobe in vitro transcription system (Promega). The RNase protection assay was conducted with the RPA III ribonuclease protection assay kit (Ambion) according to the instruction manual. After separation of protected fragments on a 6% sequencing gel, signals were quantified by phosphorimaging (Amersham Biosciences). The TAP-1 signal intensity was normalized by that of the GAPDH signal, which served as a loading control. The percentages of the amount of remaining TAP-1 mRNA at different time points after tetracycline was added compared with time 0 were calculated.
contained homozygous G alleles as described previously (20), both SK-MEL-19 and the breast cancer cell line SK-BR-3 were homozygous for T alleles that lack the restriction site for AflIII. To test whether this single-nucleotide replacement results in reduced promoter activity, both alleles of the TAP-1 promoter were cloned into the pGL2-basic vector that contains the luciferase reporter gene. As shown in Fig. 2b, the T allele TAP-1 promoter retained 50% of the promoter activity compared with the G allele. However, given the significant variation in transient transfection and luciferase assays, it is unclear whether the G→T change has a significant effect on TAP-1 transcription. However, both promoters were equally efficiently induced by IFN-γ/H9253 treatment, whereas the TAP-1 mRNA in the original SK-MEL-19 cell line was not induced by IFN-γ treatment (Fig. 1b). Moreover, our analysis of normal human peripheral blood lymphocyte samples revealed that both alleles were present at a high frequency, and individuals that carry either G or T alleles have equivalent cell surface HLA class I antigen expression (data not shown).

We therefore performed a nuclear run-on assay to directly evaluate the transcription of the TAP-1 gene. LMP-2 transcription, which is under the control of the same bidirectional promoter, was also evaluated. As shown in Fig. 2c, TAP-1 was transcribed at high levels in SK-MEL-19 cells under basal conditions, although IFN-γ appeared to up-regulate TAP-1 transcription somewhat. In contrast, LMP-2 was transcribed at an undetectable level but was induced to high levels by IFN-γ (Fig. 2c). The lack of LMP-2 transcription at basal condition may reflect the IFN-γ-inducible expression pattern of this gene. Taken together, the results demonstrate that a posttranscriptional defect is responsible for poor TAP-1 expression in SK-MEL-19 cells even after IFN-γ/H9253 stimulation. Numerous studies have revealed defective TAP-1 expression among tumor cells (7, 23). To our knowledge, however, this is the first example of a posttranscriptional defect of TAP-1 expression.

A Single-nucleotide Deletion Leads to Accelerated Decay of TAP-1 mRNA—A major mechanism responsible for posttranscriptional regulation of mRNA is RNA degradation, which can be prevented by CHX, a protein synthesis inhibitor of mammalian cells. It is well established that the turnover of mRNA is closely linked to the translation process and that blocking translation can stabilize mRNA, especially those mRNA with short half-lives (21–23). To test whether accelerated RNA deg-
radiation is responsible for the lack of TAP-1 in SK-MEL-19 cells, we treated the SK-MEL-19 and control HeLa cells with CHX after incubation with or without IFN-γ (1000 units/ml) for 48 h at 37°C. At different time points after the CHX was added to the cell culture, cells were harvested, and the total cellular RNA was analyzed for TAP-1 mRNA. The intensity of each band was quantified using ImageQuant 5.0 software (Amer sham Biosciences) after exposure to a phosphorimaging screen (Fig. 3). For a better comparison, TAP-1 mRNA levels were normalized to the endogenous housekeeping gene GAPDH, and the fold increase compared with non-CHX-treated cells was calculated. Under basal conditions, the TAP-1 mRNA was up-regulated 4.8-fold in SK-MEL-19 cells. After IFN-γ induction, the TAP-1 mRNA was up-regulated 25.7-fold. In comparison, CHX caused a less significant increase of TAP-1 mRNA in both IFN-γ-treated and untreated HeLa cells. Taken together, the lack of TAP-1 mRNA, the normal transcription of TAP-1, and the rescue of TAP-1 mRNA by CHX treatment suggest that the TAP-1 mRNA was rapidly degraded in the SK-MEL-19 cells. It is noteworthy that in SK-MEL-19 cells, the effect of CHX was significantly stronger when used in combination with IFN-γ. This finding cannot be fully explained by the fact the IFN-γ is a transcriptional activator for antigen presentation genes, because its effect on TAP-1 transcription is not so obvious in SK-MEL-19 cells as shown in Fig. 2c. It is likely that IFN-γ stabilized mRNA in SK-MEL-19 cells, although this possibility remains to be tested formally.

The rapid degradation of TAP-1 mRNA can be due to a genetic lesion in the TAP-1 gene. Alternatively, it is possible that the tumor cell line expresses factors that can cause TAP-1 mRNA degradation. The successful rescue of cell surface HLA class I antigen expression by wild type TAP-1 in SK-MEL-19 cells favors the first hypothesis because a wild type cDNA can be expressed in the tumor cell line. As the first step to test this hypothesis, we cloned TAP-1 cDNA from SK-MEL-19 cells that were treated with both IFN-γ and CHX as described above. All of the three clones sequenced showed a single-nucleotide deletion at position +1489 (Fig. 4a), which resides in exon 7 in the TAP-1 gene. Further analysis showed that multiple downstream premature termination codons (the closest one is at position +1555) were present due to this nucleotide deletion (two of them are shown in Fig. 4c). To confirm that the mutation was in the TAP-1 gene, we amplified exon 7 of the TAP-1 gene from the SK-MEL-19 cells by PCR. The PCR products were digested with BstI because this restriction enzyme recognizes the deletion mutant but does not recognize the wild type exon 7. Because complete digestion was obtained, it appears that the SK-MEL-19 cells are homozygous for the frameshift mutation (Fig. 4b), even though the cytogenetic analysis revealed that there are four copies of chromosome 6 present in the SK-MEL-19 cells (data not shown). We subsequently amplified exon 7 of the TAP-1 gene from 50 normal human peripheral lymphocyte genomic DNA samples by PCR and subjected the PCR products to BstI digestion. Because none of the PCR products from the 50 samples could be digested by BstI, it is most likely that the single-nucleotide deletion in SK-MEL-19 cells resulted from a somatic mutation (data not shown) and that the apparent homozygosity of the TAP-1 locus caused aneuploidy.

Premature termination codons resulting from frameshift mutation or nonsense mutation have been shown to interfere with the metabolism of many different mRNAs in mammalian cells, leading to nonsense-mediated altered RNA splicing, such as exon skipping and intron retention and/or NMD (21–23). Alternatively, a mutation may disrupt a cis-element that is necessary for mRNA stability and thereby cause RNA decay. To differentiate the two possible mechanisms, we designed another TAP-1 mutant (TAP-1 Del3) that has an in-frame 3-nucleotide deletion at the same position as TAP-1 D1489 (Fig. 5a) and compared the mRNA half-lives of TAP-1 WT, TAP-1 D1489, and TAP-1 Del3 cDNA constructs was conducted using the antisense transcripts as probe. a, depiction of the Tet-Off system and the pBIEGFPTAP1 constructs containing TAP-1 WT, TAP-1 D1489, and TAP-1 Del3 used to measure the mRNA half-lives. The nucleotide sequences around the mutation site in the cDNA constructs are denoted. b, decay kinetics of the TAP-1 WT, TAP-1 D1489, and TAP-1 Del3 mRNA. Means ± S.D. of four experiments are presented. The half-lives of the mRNA were determined by manual best-fit regression analysis. c, RNase protection assay to demonstrate the accelerated decay of TAP-1 D1489 and TAP-1 Del3 message compared with that of the wild type TAP-1. The antisense probe protects a 345-nucleotide TAP-1 mRNA. One representative assay for each TAP-1 construct is shown.
stable than that of the TAP-1 D1489 (t1/2 = 3.5 h). However, the mRNA derived from TAP-1 Del3 (t1/2 = 2.7 h), which has an in-frame 3-nucleotide deletion, was degraded at least as fast as that of TAP-1 D1489. Because the TAP-1 Del3 mRNA has no premature termination codon downstream to the deletion, the accelerated decay in mutant TAP-1 mRNA is most likely through mechanisms other than NMD. It is more likely that the mutation disrupts a cis-element critical for the stability of TAP-1 mRNA. Whereas few cis-elements that help to stabilize mRNA have been reported, at least two have been reported by others (24, 25) and our group (26). Preliminary analysis showed no similarity between the region surrounding the mutation and the previously reported cis-element.

Nevertheless, the NMD is a well-conserved cellular surveillance mechanism. Whereas our work revealed a non-NMD mechanism for TAP-1 mRNA degradation, it is still possible that mRNA derived from the endogenous mutant TAP-1 gene can also be degraded by NMD. It has been shown recently that several criteria have to be met for the pathway to degrade a premature termination codon-containing message. First, at least one downstream spliceable intron is required for optimal NMD (27–29). The intron is thought to help recruit NMD factors, such as hUpf3, to the mRNA via the spliceosome (30–32). Second, the premature termination codon should be at least 45–55 nucleotides away from the next spliceable intron (27, 28). The lack of introns in our constructs may have prevented us from revealing NMD in TAP-1 mRNA degradation. However, intronless premature termination codon-containing HEXA mRNA was shown to be subject to NMD, although at a lower efficiency than that seen when multiple downstream introns are present (33). In preliminary studies, when we made pBI-EGFP/TAP1 constructs with intron 7 or 8, the results also failed to support a role for NMD in degradation of the mutated TAP-1 mRNA (data not shown).

Posttranscriptional regulations of other genes involved in antigen presentation have been reported previously (34). The increased turnover of HLA-C heavy chain mRNA has been suggested to contribute to the low level of HLA-C surface expression (34). Our work shows that mutations in the TAP-1 gene in a tumor cell line can modulate its mRNA stability. This mechanism may be exploited by tumors to evade host immunity.

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