

Immune Escape of Melanoma: First Evidence of Structural Alterations in Two Distinct Components of the MHC Class I Antigen Processing Pathway¹

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Abstract

Sequence analyses of the transporter associated with antigen processing (TAP) in tumor cell lines with deficient MHC class I surface expression identified a bp deletion at position 1489 near the ATP-binding domain of *Tap1*, causing a frameshift in one melanoma cell line. The impaired TAP1 protein expression was associated with deficient TAP2 protein expression, peptide binding, translocation, and MHC class I surface expression. Stable TAP1 gene transfer reconstitutes the described defects, whereas lysis by HLA-A2-restricted CTLs was still abrogated. This was attributable to a 2-bp insertion at position 890 in the *HLA-A2* gene and was corrected after HLA-A2 cotransfection. This study describes for the first time mutations in two distinct components of the MHC class I antigen processing pathway, suggesting an immune selection against CTLs recognizing both TAP-dependent and -independent T-cell epitopes.

Introduction

Antigen presentation in the context of the MHC class I molecules to CD8⁺ CTLs requires the ATP-dependent TAP³ consisting of the subunits TAP1 and TAP2 (1). TAP transports cytosolic peptides into the endoplasmic reticulum (2). The crucial role for TAP in antigen processing was underscored by the characterization of mutant cell lines with defects in the presentation of antigenic peptides (3), by the existence of viral proteins abrogating TAP function (4), and by the analysis of TAP1^{-/-} mice (5).

In human tumors of distinct histology, changes in the expression and/or function of the MHC class I antigens were frequently found and may provide malignant cells with mechanisms to escape from T-cell recognition and destruction (6–9). The deficient MHC class I surface expression can be caused by total, locus, and allele-specific loss or down-regulation of MHC class I antigens attributable to distinct molecular mechanisms (6, 7). Loss of MHC class I antigens is often attributable to structural alterations of components of the MHC class I APM. In addition, deficiencies in MHC class I surface expression could also be caused by defects in the expression and/or function of different components of the MHC class I antigen processing pathway.

Indeed, down-regulation of MHC class I antigen expression was frequently associated with impaired TAP expression in many tumors (10). Until now, limited information exists about the underlying

molecular mechanisms of these TAP deficiencies. Thus far, only a single structural alteration in TAP has been described in a lung cancer cell line that is attributable to a functionally defective allele of *Tap1*, resulting in the loss of proper MHC class I antigen processing and presentation (11). To investigate the underlying mechanisms of MHC class I abnormalities, a series of melanoma and renal cell carcinoma cell lines with deficient MHC class I surface expression was analyzed for structural integrity, expression, and function of MHC class I antigens and the peptide transporter subunits.

Materials and Methods

Cell Lines and Culture. buf1280 cells were established from a metastatic lesion of a melanoma patient expressing the HLA phenotype HLA-A2, -B51, -CW16, -DR4, -DR9, and -DQ3.

Stable Transfection. Transfections of buf1280 cells were carried out by lipofection using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. For transfection, wt TAP1 and HLA-A2 expression vectors were used (12, 13). Cells were selected in medium supplemented with 500 µg/ml G418 (Roche Molecular Biochemicals, Mannheim, Germany).

Genomic and RT-PCR. For genomic PCR, 1×10^4 cells were resuspended in 20 µl of H₂O, incubated for 10 min at 95°C, followed by an incubation with 10 µg of proteinase K for 1 h at 55°C. Then, PCR was directly performed using 50 pmol of each primer and the following conditions: 95°C for 1 min, annealing temperature for 1.5 h and 72°C for 1.5 h. One-step RT-PCR was performed using the Titan kit as described by the manufacturer (Roche, Mannheim, Germany).

Membrane Preparation and Peptide Binding Assays. Cells were thawed on ice in 10 mM Tris-buffer (pH 8.0) supplemented with protease inhibitors and 1 mM DTT and homogenized with a glass homogenizer. Lysed cells were centrifuged at $1000 \times g$ for 10 min. For collection of crude membranes, supernatant was centrifuged at $200,000 \times g$ for 30 min, and pellets were resuspended in PBS. For peptide binding, the peptide RRYQKSTEL was labeled by the chloramine-T method using 1.5 nmol of peptide and 100 µCi of ¹²⁵I. To determine the amount of functional TAP, the membranes were incubated with radiolabeled peptide (200 nM) in a total volume of 50 µl in PBS, 5 mM MgCl₂ 15 min on ice. Unbound peptide was removed by adding 500 µl of PBS, 5 mM MgCl₂ and centrifuged for 8 min at $20,000 \times g$. The supernatant was discarded, and the washing procedure was repeated once. The membrane-associated radioactivity was directly quantified by gamma counting.

Peptide Transport Assay. The peptide translocation experiments were performed as described earlier (14) using the ¹²⁵I-labeled peptides #63 (RY-WANATRI) and #600 (TNKTRIDGQY). Briefly, 2.5×10^6 cells were permeabilized with 2 IU streptolysin O (Wellcome Reagent Ltd., Beckenham, United Kingdom) in 50 µl of incubation buffer for 5 min at 37°C, followed by the addition of 10 µl of ATP (10 mM; Roche Diagnostics, Mannheim, Germany) and 2.5 µl of radioiodinated peptides (0.5 µM) in a final volume of 100 µl and incubated at 37°C for 20 min. Subsequent to cell lysis in 1% NP40 (Sigma Chemical Co., Deisenhofen, Germany), glycosylated peptides were recovered by Concanavalin A-Sepharose beads (Pharmacia, Uppsala, Sweden) overnight. The radioactivity associated with the beads was determined by gamma counting, and translocation efficiencies were calculated as described.

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³ The abbreviations used are: TAP, transporter associated with antigen processing; wt, wild type; RT-PCR, reverse transcription-PCR; mAb, monoclonal antibody; NBD, nucleotide-binding domain.

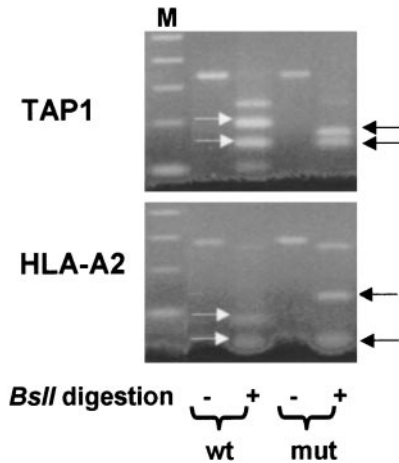


Fig. 1. Genomic PCR of mutant (*mut*) and wt TAP1. *a*, amplification products obtained by genomic PCR from the wt and *mut Tap1* gene were tested for the presence of the distinct *BstI* cleavage pattern generating in the mutated *Tap1* three fragments and two fragments in the wt *Tap1* gene. Lane 1, 100-bp molecular weight marker; Lane 2, uncut PCR product from wt *Tap1* (326 bp, cell line MZ1257); Lane 3, *BstI*-digested PCR products of wt *Tap1* (sizes 137 and 191 bp); Lane 4, uncut PCR product from *mut Tap1* (326 bp, cell line buf1280); Lane 5, *BstI*-digested PCR products of *mut Tap1* [sizes 22 (not visible) 68 and 137 bp]. *b*, amplification products of the *HLA-A2* gene were tested for the presence of the different *BstI* digestion profile, resulting in six fragments of the mutated *HLA-A2* and eight of the wt *HLA-A2* gene. Lane 1, 100-bp molecular weight marker; Lane 2, uncut PCR product from wt *HLA-A2* (391 bp, cell line MZ1257); Lane 3, *BstI*-digested PCR products of wt *HLA-A2* [sizes 7, 22, 30, (not visible) 44, 48, 51, 89, and 90 bp]; Lane 4, uncut PCR product from *mut HLA-A2* (391 bp, cell line buf1280); Lane 5, *BstI*-digested PCR products of *mut HLA-A2* [sizes 7, 22, 30 (not visible), 48, 51 and 138 bp].

Western Blot Analysis. For Western blot analysis, 20 μ g protein/lane obtained from crude membrane preparation were separated by a 10% SDS polyacrylamide gel. Proteins were blotted onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and subsequently identified using specific mAbs directed against the human TAP1 (mAb 148.3 and TM6) and the human TAP2 (mAb 429.3) protein (15, 16). Western blots were developed by secondary antibodies and enhanced chemiluminescence (ECL; Amersham Pharmacia, Freiburg, Germany).

Flow Cytometry. For indirect immunofluorescence analysis, 5×10^5 cells were incubated for 30 min on ice using the anti-MHC class I antibody HLA-ABC-FITC- and the anti-HLA-A2-specific mAb mA2.1 and then washed twice with PBS. HLA-A2-stained cells were further incubated with a FITC-conjugated goat-antimouse immunoglobulin (Coulter/Beckman, Krefeld, Germany) as a secondary antibody for an additional 30 min at 4°C. Upon washing with PBS, 10,000 viable cells were analyzed after gating on forward and side scatter on a flow cytometer (Coulter Epics XL MCL software; Coulter Epics XL MCL System II 3.0; Beckman/Coulter).

Cytolytic Assay of Tumor Cells and TAP1 Transfectants. buf1280 cells and its TAP1- and/or HLA-A2-transfected derivatives were used as targets for HLA-A2-restricted tyrosinase-specific CTLs using the standard chromium release assay (17). Cells were infected with 10 units/cell of vaccinia-tyrosinase virus and incubated for 3 h at 37°C before cells were labeled with ^{51}Cr prior to an additional incubation period at 37°C (17). Twelve h after infection, a standard chromium release assay was performed. Briefly, ^{51}Cr -labeled targets were mixed with different concentrations of effectors in triplicates using E:T ratios ranging between 30:1 and 1:1 and then incubated for 4 h at 37°C. Supernatants were then harvested, and radioactivity was determined in a gamma counter. The percentage of specific lysis was calculated as $100 \times (\text{Ex-}$

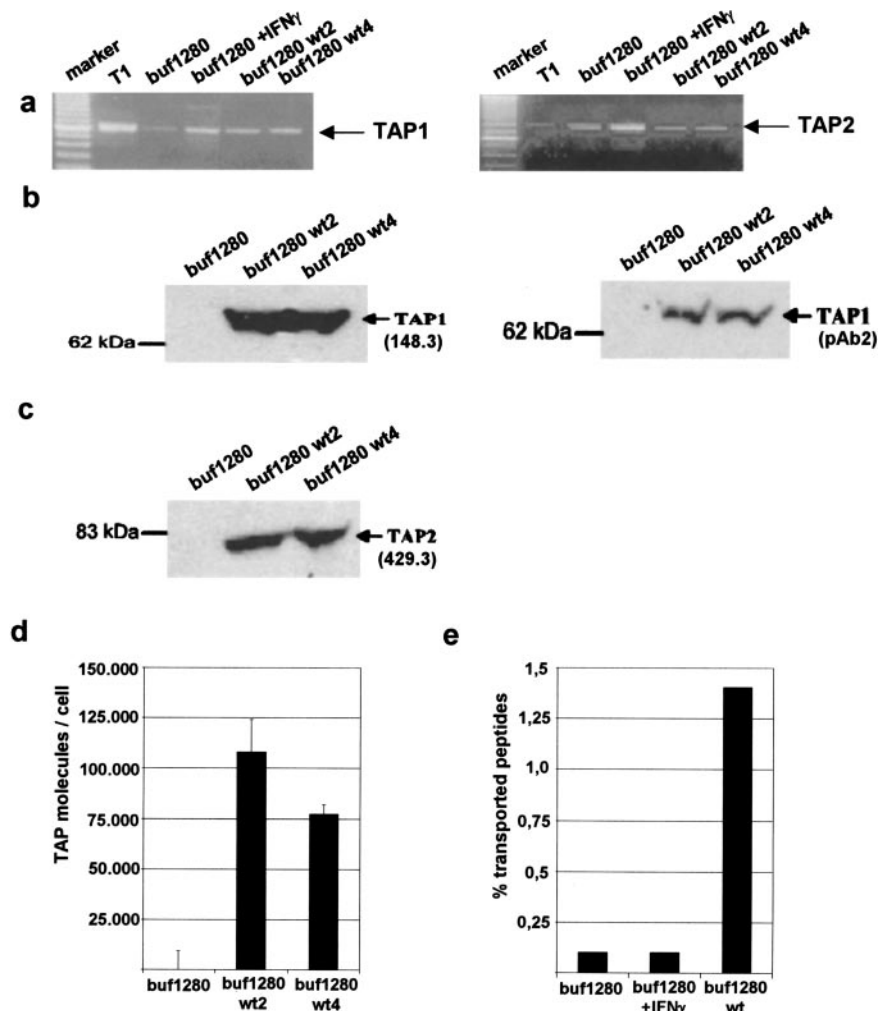


Fig. 2. Expression and function of TAP in TAP1⁻ and TAP1⁺ buf1280 cells. *a*, RNA expression of both TAP1 and TAP2 in the buf1280 cell line and control cell line is shown. Expression of both subunits is up-regulated by IFN- γ . *b*, using two different TAP1-specific antibodies, no TAP1 protein expression could be detected in parental buf1280 cells, whereas wt *Tap1* gene transfer results in wt TAP1 protein expression, as demonstrated in two TAP1 transfectants (*wt2* and *wt4*). *c*, the cell line buf1280 expressed TAP2 protein only after *Tap1* gene transfer. *d* and *e*, the restoration of TAP function was determined in untreated and IFN- γ treated TAP1⁻ and TAP1⁺ buf1280 cells using peptide binding (*d*) and translocation (*e*). Bars, SE. Lack of TAP function was found in TAP1⁻ buf1280 cells, whereas *Tap1* gene transfer reconstitutes TAP function in the TAP1 wt transfectants.

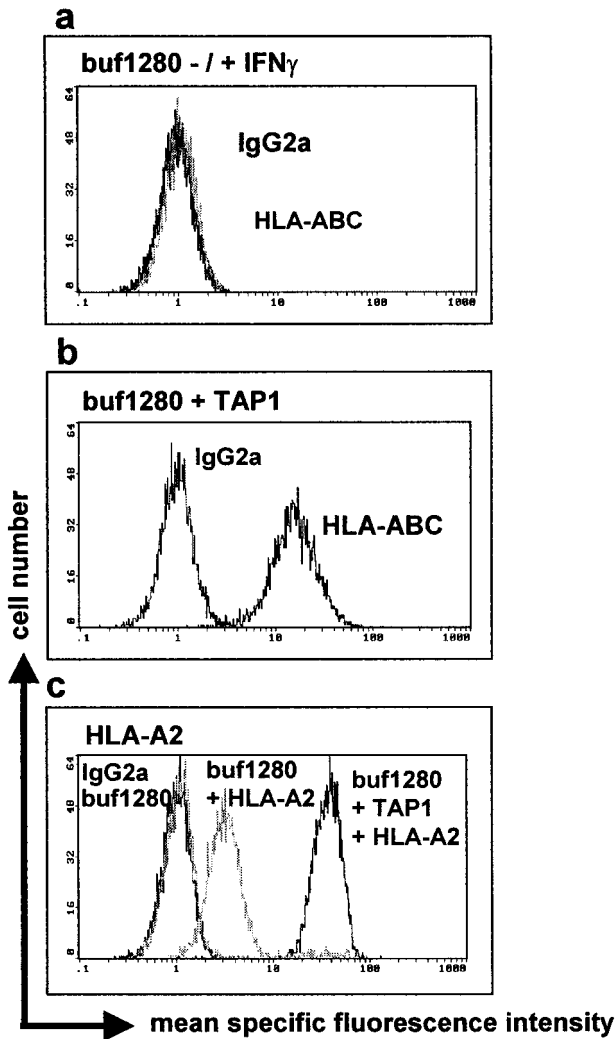


Fig. 3. Correction of MHC class I surface expression by TAP1 gene transfer. Untreated and IFN- γ -treated buf1280 cells exhibit deficient MHC class I surface expression (a), which was partially restored upon *Tap1* gene transfer (b). The expression of HLA-A2 was not restored after *Tap1* gene transfer because of an insertion of 2 bp in the *HLA-A2* gene but was corrected by *HLA-A2* gene transfer (c).

perimental release - spontaneous release)/(Maximal release - spontaneous release).

Results and Discussion

Analysis of the Structural Integrity of TAP in Tumor Cell Lines. A panel of human tumor cell lines obtained from primary and metastatic melanoma and renal cell carcinoma were analyzed for structural abnormalities in the *Tap1* gene using genomic and RT-PCR followed by direct sequencing of respective amplification products. Sequence analyses of independent RT-PCR and genomic PCR products of 15 different tumor cell lines demonstrated, with the exception of the cell line buf1280 established from a metastatic melanoma lesion, the lack of sequence alterations in the samples analyzed. In contrast, buf1280 cells exhibit a bp deletion at position 1489 in the *TAP1* gene, which occurred within the first defined fold of the NBD, resulting in a frameshift and early stop codon. The existence of this alteration was independently confirmed by *BsII* digestion of uncloned genomic PCR products, because this deletion causes a distinct restriction enzyme cleavage pattern of wt and mutated *Tap1* (Fig. 1a).

Deficient Expression and Function of TAP Attributable to TAP1 Mutation. To evaluate the functional consequences of the TAP1 mutation in buf1280 melanoma cells, the expression of the TAP

subunits, the peptide transport rate, as well as the level of MHC class I surface antigens was determined. RT-PCR analysis revealed TAP1 and TAP2 mRNA expression in the buf1280 cell line (Fig. 2a), whereas neither TAP1 nor TAP2 protein expression were detectable in these cells (Fig. 2, b and c). The impaired TAP protein expression was also accompanied by an inhibition of both peptide binding and peptide transport (Fig. 2, d and e). In addition, flow cytometric analysis using the anti-HLA class I antibody HLA-ABC-FITC revealed a lack of constitutive and IFN- γ -inducible MHC class I surface expression in the buf1280 cells (Fig. 3a). Thus, the bp deletion in the *Tap1* gene abrogates TAP protein expression and function, which is accompanied by MHC class I abnormalities.

Correction of the TAP-deficient Phenotype by TAP Gene Transfer. Stable gene transfer of wt *Tap1* into the melanoma cell line reconstituted the expression of both TAP subunits, the TAP1/TAP2 complex formation and the function of the peptide transporter as measured by peptide binding and peptide translocation into the endoplasmic reticulum (see Fig. 2, b-e). Furthermore, restoration of TAP function by *Tap1* gene transfer was accompanied by the expression of high levels of total MHC class I surface antigens on these cells (Fig. 3a).

CTL-mediated Immune Escape Attributable to Combined Structural Alterations of the TAP1 and HLA-A-2 Genes. To define whether there exists a correlation between the level of MHC class I surface expression and immune recognition, uninfected and MVA-tyr-infected TAP1⁻ and TAP1⁺ buf1280 cells were used as targets for HLA-A2-restricted tyrosinase-specific CTLs. Although total MHC class I surface expression was corrected by wt *TAP1* gene transfer, no recognition and killing of both parental TAP1⁻ and TAP1⁺ buf1280 cells were found, whereas the tyrosinase expressing, HLA-A2⁺ melanoma cell line NA8 was efficiently lysed by these CTLs (Fig. 4). The lack of susceptibility to tyrosinase-specific lysis was attributable to abrogated expression of HLA-A2 surface antigens (Fig. 3b). Sequence analyses of genomic and RT-PCR products demonstrated a 2-bp insertion at position 890 in the *HLA-A2* gene, which was further confirmed by a distinct *BsII* digestion pattern of the genomic PCR products from parental buf1280 cells when compared with that of respective control cells (Fig. 1b). The impaired HLA-A2 expression could be overcome by wt *HLA-A2* gene transfer (Fig. 3c). The TAP1⁺, HLA-A2⁺ buf1280 cells, but not the buf1280 HLA-A2⁺ variants, were efficiently recognized by the HLA-A2-restricted tyrosinase-specific CTLs, demonstrating that the processing and presentation of HLA-A2-restricted, tyrosinase-specific peptides could be re-

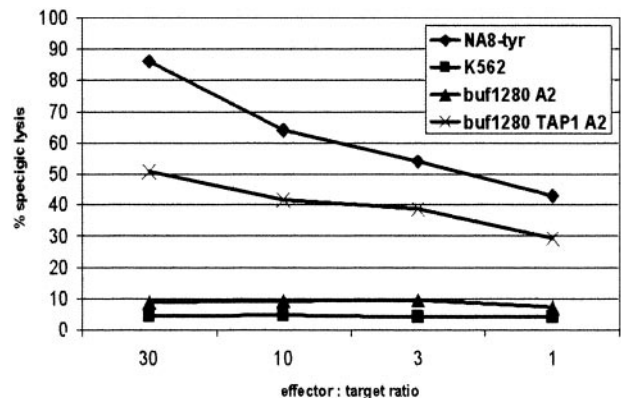


Fig. 4. Induction of CTL-specific lysis after *TAP1* and *HLA-A2* gene transfer. The TAP1⁻, HLA-A2⁺ buf1280 cells were not lysed by tyrosinase-specific CTLs, whereas the TAP1⁺, HLA-A2⁺ buf1280 cells were efficiently recognized with a 50% lysis. The cell line NA8-tyr served as positive control, and the cell line K562 served as a negative control.

stored by cotransfection of both the wt *Tap1* and *HLA-A2* genes into the buf1280 melanoma cell line (Fig. 4).

Thus, we here describe for the first time the simultaneous occurrence of structural alterations in two components of the MHC class I APM, a 1-bp deletion within the first defined fold of the NBD of the *Tap1* subunit and a 2-bp insertion within the *HLA-A2* gene that are responsible for the lack of antigen processing and presentation as well as for MHC class I surface expression abnormalities. These data further suggest that the buf1280 cells evade the efficient CTL-mediated immune recognition by abrogating the presentation of both TAP-independent and TAP-dependent peptides in the context of the MHC class I molecules.

Mutations in the *Tap* genes have been found to severely affect MHC class I surface expression (18). To the best of our knowledge, no naturally occurring homozygous deletion resulting in the inactivation of TAP protein has been demonstrated in any murine and human tumor cell line. Chen *et al.* (11) demonstrated an R-to-Q substitution at residue 659 in the TAP1 subunit. This codon is located in the cytosolic domain of the ATP-binding region of the *Tap1* gene. These authors suggest that the ATP-binding ability of this novel allele may be affected by the substitution of an arginine to a glutamine. Recently, our group has identified specific sequences in the putative peptide binding domains that are essential for proper antigen processing without affecting the ATP-binding capability (19). These results argue for an influence of the conformation of these regions for proper peptide binding, suggesting that structural alterations near the NBD might negatively interfere with these properties. The TAP1⁻ melanoma cell line buf1280 lacks TAP2 protein expression, although TAP2 mRNA was transcribed and no sequence alteration was detected. Because *Tap1* gene transfer also restores TAP2 protein expression, it is suggested that wt TAP1 stabilizes TAP2 protein and that both TAP subunits are coordinated regulated. Thus, a novel mechanism of TAP dysfunction and a newly identified phenotype of TAP mutations in human tumors have been identified.

Tumor cell lines of distinct origin often exhibit impaired expression and function of TAP, which could be often restored by IFN- γ treatment (10). In contrast to these studies, the TAP deficiency we defined in buf1280 cells cannot be overcome by IFN- γ treatment caused by a structural alteration in *Tap1*. Transfection of the wt *Tap1* gene restored the expression and ability to process and present antigens in the context of MHC class I molecules, demonstrating that this deletion is directly accompanied by deficient MHC class I surface expression and at least a TAP-dependent dysfunction of the antigen processing. The lack of induction of the HLA-A2-restricted, CTL-mediated recognition of the TAP1⁺ melanoma cell line was attributable to an additional structural alteration in the *HLA-A2* gene of buf1280 cells and enables their escape from the TAP-independent immune response, because peptides also derived from signal sequences cannot be presented (20). The concomitant mutations in both *TAP1* and *HLA-A2* genes implicate an immune selection mediated also by CTL recognizing TAP-independent epitopes. Until now, a number of mutations have been reported in the *HLA-A2* gene ranging from point mutations, substitutions to bp insertions (7, 9), but the 2-bp insertion at position 890 of the *HLA-A2* gene demonstrated in buf1280 cells has not been described thus far. Thus, our data significantly extend the observations of others demonstrating: (a) a coordinated regulation of both TAP subunits; (b) a novel TAP mutant phenotype; and (c) the first description of a combined structural alteration of two distinct APM components

causing a concomitant loss of the TAP-independent and TAP-dependent antigen presentation function in human tumors.

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