Brief Definitive Report

Strictly Transporter of Antigen Presentation (TAP)–dependent Presentation of an Immunodominant Cytotoxic T Lymphocyte Epitope in the Signal Sequence of a Virus Protein

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Summary

Peptides presented by major histocompatibility complex (MHC) class I molecules are derived from intracellularly synthesized proteins. Cytosolic proteins are fragmented into peptides, which are subsequently transported via the transporter of antigen presentation (TAP) into the endoplasmic reticulum (ER), where they bind to MHC class I molecules. We have investigated the requirements for MHC class I presentation of the immunodominant gp33 cytotoxic T lymphocyte epitope of the lymphocytic choriomeningitis virus. This epitope is located within the leader peptide of the virus glycoprotein. Such an epitope is expected to be presented in a TAP-independent manner, since it is released into the ER by signal peptidase. Taking advantage of TAP1$^{-/-}$ mice, however, we show both in vitro and in vivo that, after virus infection, the presentation of the gp33 epitope is strictly dependent on a functional TAP heterodimer. The results are discussed with respect to peptide trimming processes in the ER.

Peptides presented by MHC class I molecules are derived from endogenously synthesized proteins. The degradation of these proteins into peptides takes place in the cytosol. Recently, proteasomes have been shown to be involved in the generation of presentable peptides (1), suggesting that the pathway for generation of antigenic peptides is intimately linked to the machinery that controls the normal turnover of cellular proteins. These cytosolic peptides are transported via the ATP-dependent transporters of antigen presentation (TAP) into the lumen of the endoplasmic reticulum (ER), where they bind to newly synthesized MHC class I molecules (2, 3). The trimeric MHC–β_2-microglobulin–peptide complex is then transported to the cell surface.

The nature of the peptides bound to MHC class I has been studied extensively (4). Such peptides have a length of 8–10 amino acids and display allele-specific anchor residues; longer peptides have only rarely been eluted from MHC class I molecules. The questions of in which compartment peptides are trimmed to their final length and whether there is a preselection of presentable peptides via proteasome cleavage and TAP transporter specificities are current matters of debate. The TAP translocators appear to have a preference for peptides with a length of 8–15 amino acids displaying a hydrophobic COOH-terminal end, thus matching with the overall binding preferences of MHC class I (5, 6). Nevertheless, a large fraction of the TAP-translocated peptides is longer than 8–9 amino acids. In addition, MHC class I–restricted epitopes can only be detected in cells expressing the corresponding restriction element (7). From such data, peptide trimming processes within the ER have been postulated. In the ER, a source of peptides of rather heterogenous length are the leader peptides derived from signal sequences of secretory and membrane-bound proteins. These peptides enter the ER in a TAP-independent manner, since they are cleaved from nascent polypeptides by the signal peptidase (8). Indeed, the presentation of signal sequence–derived epitopes on MHC class I has been demonstrated in TAP mutant cell lines (9, 10). Based on such findings, which support the concept of peptide trimming in the ER, a second, TAP-independent pathway of antigen presentation has been postulated (9). However, the contribution of this pathway to antigen presentation under physiological conditions has remained elusive, and the virtual absence of CD8$^+$ T cells in TAP1$^{-/-}$ mice argues against a major role of this pathway (11). In this study, we analyze the presentation of an immunodominant CTL epitope located within the signal sequence of

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the glycoprotein (GP) of the lymphocytic choriomeningitis virus (LCMV). Contrary to expectations (12), we show that this H-2^b–restricted epitope cannot be presented in a TAP-independent manner. If these data do not indicate unexpected transport pathways of some leader peptides avoiding the ER, they may suggest only limited trimming activity in the ER, arguing against a major role of a TAP-independent pathway of antigen presentation.

Materials and Methods

Animals and Viruses. The P14 TCR–transgenic and the TAP1$^{-/-}$ mice have been described elsewhere (11, 13). 129 control mice were bred locally. The 327 founder strain was backcrossed to B6.C-H-2^{bm13} (kindly provided by Dr. C. J. Melief, Academisch Ziekenhuis Leiden, Leiden, Netherlands) (14). The MHC haplotype was determined using the anti-D^b antibody H141-35 (15). The WE strain of LCMV was originally obtained from Dr. F. Lehmann-Grube (Heinrich Pelte Institut, Hamburg, Germany) (16). Virus stocks were diluted in MEM supplemented with 2% FCS.

Flow Cytometry. ~10^6 cells were incubated for 20 min at 4°C with the various antibodies in 100 μl volume. Cells were washed in BSS, 2% FCS, 0.03% NaN_3. For double staining of P14 TCR–transgenic splenocytes, the following antibodies were used: anti-CD8-FITC (clone 53; GIBCO BRL, Gaithersburg, MD) and anti-Vα2-PE (01655A; PharMingen, San Diego, CA). LCMV-infected fibroblasts were first incubated with normal rat serum and were subsequently stained with the anti-LCMV glycoprotein antibody KL25 (17) or mouse serum as control. KL25 was detected with goat anti–mouse IgG1-FITC (Southern Biotechnology Associates, Birmingham, AL). Cells were analyzed on a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Cytotoxic and Proliferative Assays. LCMV-infected peritoneal macrophages were obtained from mice given 1 ml thioglycolate intraperitoneally on day −6 and 500 PFU LCMV-WE on day −4. Cells were peptide loaded by incubating them for 2 h with 10^{-8} M gp33 peptide. Stimulatory macrophages were irradiated with 2,000 rad. For in vitro stimulation of CTLs, 10^5 macrophages were incubated in 24-well plates for 3 d with 2×10^6 P14^{bm13} splenocytes in IMDM, 10% FCS, 10^{-5} M β-mercaptoethanol. Stimulated spleen cells were resuspended in 50% of culture volume and were added in serial threefold dilutions to the target cells. CTL activity was measured on EL4 cells in a 5-h standard chromium release assay, as described elsewhere (18). For measurement of the proliferative response, stimulatory macrophages were titrated to 2×10^5 P14^{bm13} splenocytes in 96-well plates; the culturing conditions were as above. After 36 h, cells were pulsed for 8 h with [^{3}H]thymidine (50 μl; 25 μCi/ml).

Embryonic fibroblast target cells were prepared as follows: Mouse embryos (day 15–20) were trypsinized, and cells from the supernatant fraction were cultured in IMDM, 10% FCS. After two to three passages, cells were infected with LCMV for 48 h with a multiplicity of infection of 10^{-3}, or labeled for 2 h with 10^{-8} M gp33 peptide. Their sensitivity as CTL targets was assessed in a standard 5-h chromium release assay, using D8 LCMV-WE–immune C57BL/6 splenocytes.

Adoptive Transfer Experiment. Before transfer, mice were irradiated with 450 rad. The following day, single-cell suspensions of 10^6 P14^{bm13} splenocytes in BSS were transferred intravenously. The same day, mice were primed with 2×10^4 PFU LCMV-WE intravenously. 8 d later, spleens were collected and analyzed by flow cytometry.

Results and Discussion

TAP1$^{-/-}$ Cells Are Readily LCMV Infected and Express the LCMV GP on the Cell Surface. The major H-2^b–restricted CTL epitope of the LCMV GP comprises amino acid position 33-41 (gp33) within the unprocessed precursor polypeptide (13). As a type I membrane protein, LCMV-GP contains an NH_2-terminal signal sequence, which is removed after transport of the protein into the ER. The cleavage site of the leader peptide has been determined by NH_2-terminal sequencing of the mature GP (19). It locates at position 58, and thus defines gp33 as a leader epitope. We have used this epitope to study the role of peptide trimming in the ER using TAP1$^{-/-}$ knockout mice (11). Embryonic fibroblast lines were established from TAP1$^{-/-}$ and 129 control mice and were infected with LCMV. 2 d after infection, cells were examined for surface expression of LCMV-GP by flow cytometry (Fig. 1). TAP1$^{-/-}$ and control fibroblasts showed a similarly high expression level of LCMV-GP on the cell surface, suggesting that both cell types generated comparable amounts of leader peptide in the ER. Some variability in the expression pattern could be observed from experiment to experiment, for reasons unknown.

TAP1$^{-/-}$ Cells Do Not Present a Leader Peptide–derived Epitope. Two approaches were chosen to investigate whether LCMV-infected TAP1$^{-/-}$ cells are able to present the gp33 epitope in context of H-2^b. In the first set of experiments, we analyzed whether LCMV-infected TAP$^{-/-}$ embryonic fibroblasts could serve as target cells. In a standard ^{51}Cr-release assay, virus-infected and peptide-labeled fibroblasts were incubated with LCMV-immune effector cells from C57BL/6 mice (Fig. 2). The TAP1$^{-/-}$ LCMV-infected fibroblasts showed a markedly reduced susceptibility to lysis as compared with the control cells. Although lysis of primary fibroblasts is variable, which may be due to low MHC expression, the result could be repeatedly confirmed. Thus, the presentation of the leader peptide–derived gp33 epitope was greatly impaired in TAP1$^{-/-}$ fibroblasts.

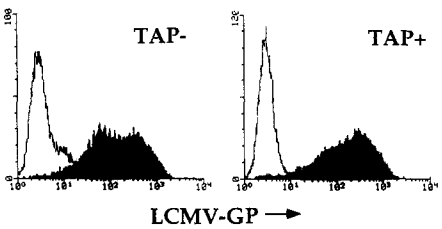

Figure 1. Cell-surface expression of the LCMV GP. TAP1$^{-/-}$ and control embryonic fibroblasts were infected with LCMV at a multiplicity of infection of 10^{-3} and were surface-stained 2 d later with the LCMV-GP–specific antibody KL25 (*filled histograms*). Open histograms represent staining controls.

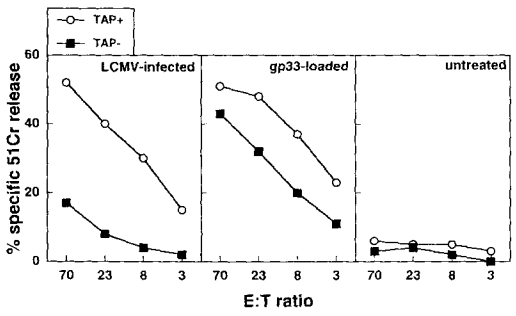

Figure 2. LCMV-infected fibroblasts as target cells. Embryonic fibroblasts from TAP1$^{-/-}$ (*filled squares*) and TAP^{+} 129 control mice (*open circles*) were either LCMV infected, gp33 peptide loaded, or left untreated. After labeling with ^{51}Cr, their capacity to serve as target cells was measured in a standard chromium release assay, using LCMV immune effector cells at the indicated E/T cell ratios. Spontaneous release was <30%.

In a second set of experiments, the capacity of LCMV-infected TAP1$^{-/-}$ peritoneal macrophages to induce CTLs in vitro was assessed. Stimulatory TAP1$^{-/-}$ or control macrophages were either LCMV infected, gp33 peptide loaded, or left untreated. As responders, splenocytes from P14 TCR–transgenic H-2^{bm13} mice were used. The P14 TCR recognizes the gp33 epitope in the context of H-2D^{b} (13). This transgenic line was back-crossed to B6.C-H-2^{bm13} mice, which exhibit a mutation in their D^{b} molecule and therefore cannot present the gp33 peptide to the P14 TCR (20, 21), but it still allows positive selection of the P14 TCR. Thus, in this assay, the responder splenocytes themselves have no stimulatory capacity for the gp33 peptide. After 3 d, cultures were examined for CTL activity in a ^{51}Cr-release assay (Fig. 3). Whereas peptide-loaded TAP1$^{-/-}$ and control macrophages had the same capacity to induce CTLs, only the LCMV-infected TAP^{+} control, but not TAP1$^{-/-}$ macrophages, showed stimulatory activity. In addition, stimulation of P14 TCR transgenic splenocytes was assessed in a proliferation assay, and no stimulatory capacity of LMCV-infected TAP1$^{-/-}$ macrophages

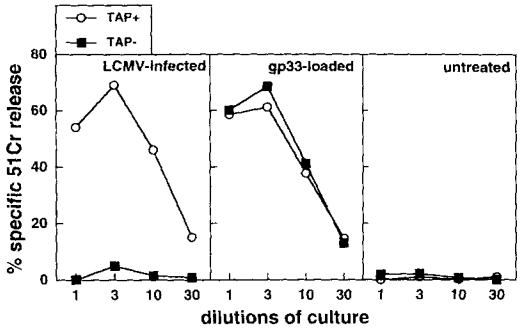

Figure 3. In vitro stimulation of CTLs. Peritoneal macrophages of TAP1$^{-/-}$ mice (*filled squares*) or TAP^{+} 129 control animals (*open circles*) were either LCMV infected, loaded with gp33 peptide, or left untreated. 10^{5} macrophages were incubated for 3 d with 2 × 10^{6} P14 TCR–transgenic H-2^{bm13} splenocytes. In a 5-h standard chromium release assay, cultures were subsequently tested for cytolytic activity against gp33-labeled or untreated EL4 target cells. Unspecific killing of untreated EL4 targets was <5%, and spontaneous release was <10%.

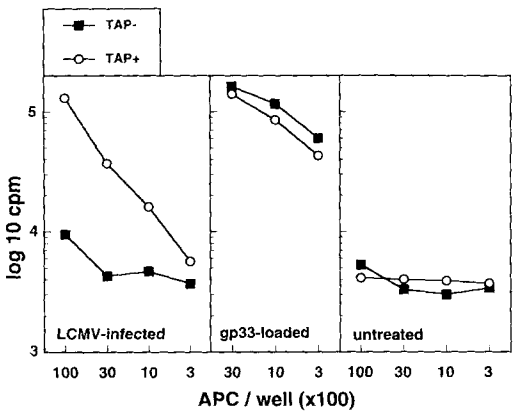

Figure 4. Proliferative response to LCMV-infected macrophages. Peritoneal macrophages of TAP1$^{-/-}$ mice (*filled squares*) or TAP^{+} 129 control animals (*open circles*) were either LCMV infected, loaded with gp33 peptide, or left untreated. Irradiated stimulatory macrophages were titrated into 96-well plates at indicated concentrations, and 2 × 10^{5} P14bm13 responder splenocytes were added. After 36 h, cells were pulsed for 8 h with [^{3}H]thymidine (25 μCi/ml) for assessment of proliferation.

was observed (Fig. 4). Thus, TAP1$^{-/-}$ macrophages were unable to present the leader peptide–derived gp33 epitope. Taken together, these in vitro experiments, using two different cell types, show that the LCMV gp33 leader epitope could not be presented in a TAP-independent manner.

No In Vivo Presentation of a Leader Peptide–derived Epitope in TAP1$^{-/-}$ Mice. The inability of TAP1$^{-/-}$ cells to present the LCMV-GP leader epitope was confirmed in an in vivo assay, in which the expansion of P14 TCR–transgenic cells was assessed after LCMV infection of TAP1$^{-/-}$ mice. Splenocytes from TCR-transgenic H-2^{bm13} mice were adoptively transferred into sublethally irradiated TAP1$^{-/-}$ and control mice. Mice were subsequently infected with LCMV, and 8 d later the expansion of the P14 TCR–transgenic T cells was analyzed by flow cytometry. Whereas, in the control mice, the transferred P14 population (CD8, Vα2) markedly increased in response to the virus infection, no expansion was observed in the TAP1$^{-/-}$ mice (Fig. 5). These data confirm the results obtained in vitro and demonstrate that no biologically relevant residual presentation of this signal sequence–derived epitope occurred in TAP1$^{-/-}$ mice.

This study takes advantage of TAP1$^{-/-}$ mice and analyzes the presentation of antigen from a noncytopathic virus, which does not usually interfere with normal cellular functions. We show that the immunodominant viral CTL epitope gp33, located within the signal sequence of the LCM virus GP, cannot be presented in a TAP-independent manner. Thus, although abundant amounts of leader peptides are generated during virus infection, as evidenced by the high GP expression on the cell surface, they fail to be processed to associate with MHC class I molecules inside the ER. However, several reports have demonstrated TAP-independent peptide loading of MHC class I molecules. These data have been generated using the TAP mutant cell lines RMA-S and T2, and fall mainly into two categories: studies analyzing the presentation of viral antigen

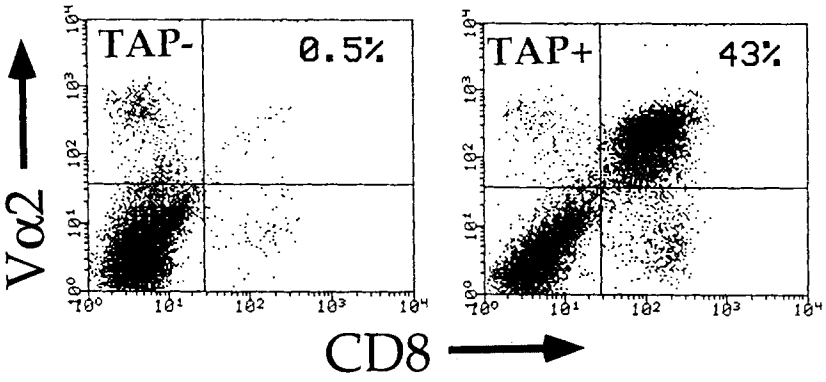

Figure 5. In vivo expansion of P14 TCR–transgenic H-2^{bm13} splenocytes after LCMV infection. $TAP1^{-/-}$ and TAP^+ 129 control mice were irradiated sublethally with 450 rad. At day 1 after irradiation, mice were adoptively transfused with 10^6 P14–transgenic H-2^{bm13} splenocytes and were simultaneously infected with 2×10^4 PFU LCMV intravenously. 8 d later, spleens were removed and examined for the expansion of the P14-transgenic cell population by two-color flow cytometry. $CD8^+$ cells were analyzed for the expression of the P14 TCR (Vα2). Indicated are percentages of double-positive cells of the total lymphocyte population.

after infection of the cell lines, and studies testing antigen presentation of recombinant gene products. Using virus-infected cells, several groups have reported class I presentation of viral antigen circumventing the defect of these cell lines (22–24). This residual presentation could be attributed to a "leakiness" of the RMA-S cell line, which still expresses the TAP1 molecule, and to the cytopathic effect of virus infection, such as vesicular stomatitis virus, on the infected cell, which may lead to a destruction of organelle structures. Recently, residual functional activity of TAP1 alone has been firmly demonstrated (25). However, using DNA constructs coding for small polypeptides released into the ER via signal peptidase, it has been shown that TAP-independent antigen presentation is possible (26, 27). This was in line with experiments in which signal sequence–derived peptides were eluted from HLA-A2 molecules of unmanipulated TAP mutant cell lines (9, 10). Since leader peptides are usually considerably longer than MHC class I–bound peptides, the question of to what extent trimming of peptides occurs in the ER was raised. In a recent study, this problem was approached systematically using peptides flanked with various spacer regions (27). In this system, aminopeptidases were identified to be responsible for the major trimming activity in the ER, whereas carboxy- and endopeptidases contributed only marginally. From this study, it appears that the COOH-terminal portion of the peptide needs to fit into the MHC class I peptide groove, and that trimming occurs primarily from the NH_2 terminus, confirming a model previously proposed by Rammensee and colleagues (4, 28).

Our analysis extends previous studies to an in vivo situation of a natural virus infection and supports the above model. The leader peptide of LCMV-GP has a total length of 58 amino acids, and the epitope is located at position 33-41 (13). Presentation of the CTL epitope derived from the leader peptide would thus, in addition to aminopeptidases, require carboxy- or endopeptidase activity. Our experiments do not yield evidence for such enzymatic activity in the ER. The exclusive TAP-dependent presentation of the gp33 epitope implies that only antigen degraded in the cytosol is available for MHC loading. Antigenic peptide could thus be derived from glycoprotein aberrantly not transported into the ER. Alternatively, leader peptides could be translocated from the ER into the cytosol for further degradation, to be then recycled back to the ER in a TAP-dependent manner. Such a pathway has recently been demonstrated in an in vitro approach (29). The immunodominance of the gp33 epitope may argue for such a recycling process. It should be noted that an alternative explanation of our results would be that the leader peptide is not released into the lumen of the ER. This possibility cannot be formally excluded. However, we consider this explanation unlikely, because signal peptidase is topologically ER luminal, and other leader sequences have been shown to be released into the ER.

In conclusion, our results indicate that, since only few epitopes can be expected to be located close to the COOH terminus of leader peptides, the contribution of a direct TAP-independent path for antigen presentation, although functional under selected experimental conditions, may only be of very limited importance in a virus infection.

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