Brief Definitive Report

Influences of Transporter Associated with Antigen Processing (TAP) on the Repertoire of Peptides Associated with the Endoplasmic Reticulum–resident Stress Protein gp96

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Summary

The endoplasmic reticulum (ER)-resident stress protein gp96 induces protective immunity and specific cytotoxic T lymphocyte (CTL) responses against antigens expressed in those cells it has been isolated from. This ability is based on peptides associated with gp96. Because gp96 is located inside the ER, our experiments address the question whether or not the repertoire of peptides associated with gp96 is influenced by the transporter associated with antigen processing (TAP). For this purpose, gp96 was isolated from cells with and without a TAP defect and used for immunization of mice. We found that for some antigens the association of peptides with gp96 required functional TAP molecules, whereas the association of peptides from other antigens was TAP independent. In the case of a TAP-dependent association of peptides with gp96, our results prove that peptide binding by gp96 in vivo occurs inside the ER and is not an artifact induced by cell lysis during the gp96 purification. The finding that some antigens can also associate with gp96 in the absence of functional TAP molecules indicates that the repertoire of peptides bound by gp96 truly reflects the entire repertoire of peptides present inside the ER and not only those peptides transported by TAP. These results, together with the earlier finding that the gp96 peptide repertoire is independent of the major histocompatibility complex molecules expressed by the cell gp96 is isolated from, give the theoretical foundation for the ability of gp96 to induce CTL responses against all kinds of intracellular antigens.

Immunizations with the stress protein gp96 have been shown to induce specific CTL responses against a variety of intracellular antigens, including viral and minor H antigens and also protective immunity against tumors from which gp96 was isolated (1–4). This ability of gp96 has been attributed to peptides derived from intracellular proteins that associate with gp96 inside the endoplasmic reticulum (ER) (5). In support of this is the finding that dissociation of bound peptides leads to abrogation of the gp96 immunogenicity (5).

The mechanism by which peptides associate with gp96 is not known but it has been postulated that peptides translocated by the transporter associated with antigen processing (TAP) are accepted by gp96 and that gp96 assists in the loading of peptides onto MHC class I molecules (6). Indeed, we were able to show recently that in streptolysin-O-permeabilized cells, peptides translocated into the ER are able to bind to gp96 in a TAP-dependent fashion (7). This leaves open the possibility that gp96 is directly involved in the loading of peptides onto MHC molecules, as might apply perhaps to those MHC molecules that do not associate with TAP, a process that can be mediated by tapasin in human cells (8, 9). On the other hand, gp96 might play a role in the handling of peptides unable to bind to MHC molecules without further modifications. The fate of such peptides, thought to represent the majority of TAP-transported peptides, is unknown but they might be subjected to trimming or degradation or to export from the ER, possibly by the Sec61 transporter. This complex has been shown to translocate glycoproteins from the ER back to the cytosol (10, 11). Interestingly, we found that gp96 is able to bind glycosylated peptides, which could be an indication that it supplies Sec61 with proteins destined for ER export (7). Using gp96 to induce cross-priming, we have shown earlier that the repertoire of peptides associated with gp96 is not influenced by the MHC molecules present in the cell but seem to reflect the complete immunological information of a cell (4). This would make gp96 an ideal candidate for the induction of immune responses, especially in those cases where the antigens or T cell epitopes have not been identified. But so far, the binding of peptides to gp96 inside the ER in intact cells has not been shown directly and the possibility that this association is induced as a result of cell lysis during the gp96 purification cannot be ex-

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cluded. This would make the binding of peptides to gp96 unpredictable, weakening the potential use of gp96 for therapeutical applications. Therefore, we decided to investigate the influence of TAP on gp96 peptide association and thereby identify the compartment where gp96 binds peptides. We isolated gp96 from RMA and TAP2-defective RMA-S cells expressing β-galactosidase (β-gal) and several minor H antigens and tested the ability of these different gp96 preparations to induce specific CTL responses.

Materials and Methods

Mice and Antibodies. BALB/c, B10.D2, BALB.B, 129/Sv, and C57BL/6 mice were obtained from Charles River WIGA (Sulzfeld, Germany) and were maintained in the animal facilities at the Institute for Cell Biology. Antibodies to gp96 (anti-grp94, SPA-850, clone 9G10) were obtained from Stressgen Biotechnologies Corp. (Victoria, BC, Canada).

Purification of gp96. gp96 was purified as described (4). In brief, P815, P13.1, RMA, RMA-S cells, or their β-gal transfectants were grown in roller bottles to generate a 60-ml cell pellet. This pellet was homogenized in 200 ml of hypotonic buffer (30 mM $NaHCO_3$, 0.5 mM PMSF, pH 7.1) and separated from the supernatant by a 100,000 *g* centrifugation. The supernatant was applied to a Con A–Sepharose column and eluted with PBS containing 1 mM $MgCl_2$, 2 mM KCl, and 6% α-methyl–mannoside. The eluate was dialyzed against 5 mM phosphate buffer, pH 7.0, and separated on FPLC using a MonoQ column (5/5, Pharmacia) with a NaCl gradient from 0 to 1 M. Fractions were tested in SDS-PAGE and Western blot analysis using a mAb specific for gp96. Only fractions containing gp96 and no other detectable contaminating proteins were pooled and concentrated using centricon 10 microconcentrators (Amicon). The approximate concentration was determined by measuring the OD at 280 nm using an extinction coefficient of 1.0.

Immunization of Mice and Generation of CTL. 8- to 10-wk-old female BALB/c and 129/Sv mice were immunized intraperitoneally with 10^7 irradiated C57BL/6 spleen cells (33 Gy) in 300 μl PBS or with 30 μg gp96 purified from different cell lines in 300 μl PBS. 10 d later, spleens were removed and the splenocytes were stimulated with irradiated (33 Gy) spleen cells from B10.D2 or C57BL/6 mice or synthetic β-gal peptide (TPHPARIGL; 1 μM) for 5 d.

Cell Culture and CTL Assays. RMA, RMA-S, P815, and P13.1 (12) cells were cultured in RPMI-1640 containing 10% FCS, 2-ME, L-glutamine, and antibiotics. CTL lines were generated by weekly restimulations with irradiated spleen cells from B10.D2 or C57BL/6 mice and synthetic peptides, if applicable, as described (13). CTL assays were performed as described (13) using RMA, RMA-S, P815, P13.1, and Con A blasts of spleen cells as target cells. Con A blasts were generated as described (14).

β-Gal Transfection and Determination of β-Gal Expression. RMA and RMA-S cells were cotransfected with 2 μg of the plasmid pCH110 (Pharmacia) expressing the *Escherichia coli*–derived β-gal under the control of the SV40 promotor and 10 μg of the plasmid pBJ1neo to allow G418 selection of the transfectants. Transfectants were selected with 1 mg/ml G418 (GIBCO BRL, Gaithersburg, MD). Surviving cells were cloned and tested for their expression of β-gal by their ability to cleave X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Cleavage of X-gal was determined by incubating serial dilutions of an 1% NP-40 cell lysate of 10^6 transfectants with 2.5 μg X-gal in 100 μl PBS at 37°C and measuring the absorbance at 620 nm after 8 h. Clones with comparable β-gal activity were selected and expanded for the purification of gp96.

Results

Generation of β-Gal–specific CTL with gp96 from β-Gal Transfectants Expressing Functional or Nonfunctional TAP Molecules. To test the hypothesis that peptide binding to gp96 occurs inside the ER we transfected RMA and RMA-S cells with E. coli–derived β-gal resulting in cytosolic expression of the protein (12). β-gal–transfected RMA and RMA-S cell clones, called R/gal and RS/gal and expressing similar levels of the protein were selected (Fig. 1) and expanded for the purification of gp96 according to standard protocols (4). BALB/c mice were immunized with these gp96 preparations and as a control with gp96 from P815 or P13.1, the β-gal transfectant of P815. As shown in Fig. 2 *A*, only the immunization with gp96 from P13.1 but not P815 induces a CTL response against P13.1 or peptide-incubated P815 cells, underlining the specificity of the gp96 immunization. As shown in Fig. 2 *B*, the induction of β-gal–specific CTL requires the presence of functional TAP molecules, because only the gp96 preparation from R/gal but

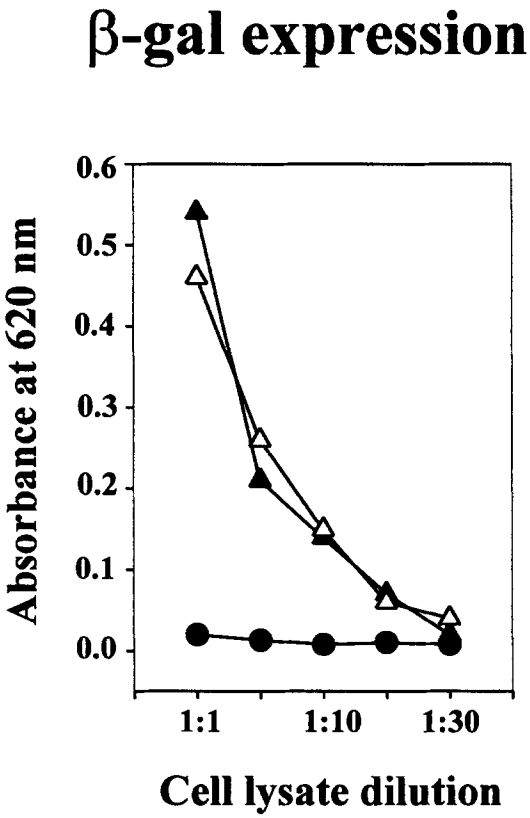

Figure 1. Determination of β-gal expression in R/gal and RS/gal transfectants. Serial dilutions of NP-40 lysates of R/gal (*open triangles*) and RS/gal (*closed triangles*) transfectants or untransfected RMA cells (*closed circles*) were incubated with the chromogenic substrate X-gal and enzyme activity was determined by measuring the absorbance at 620 nm.

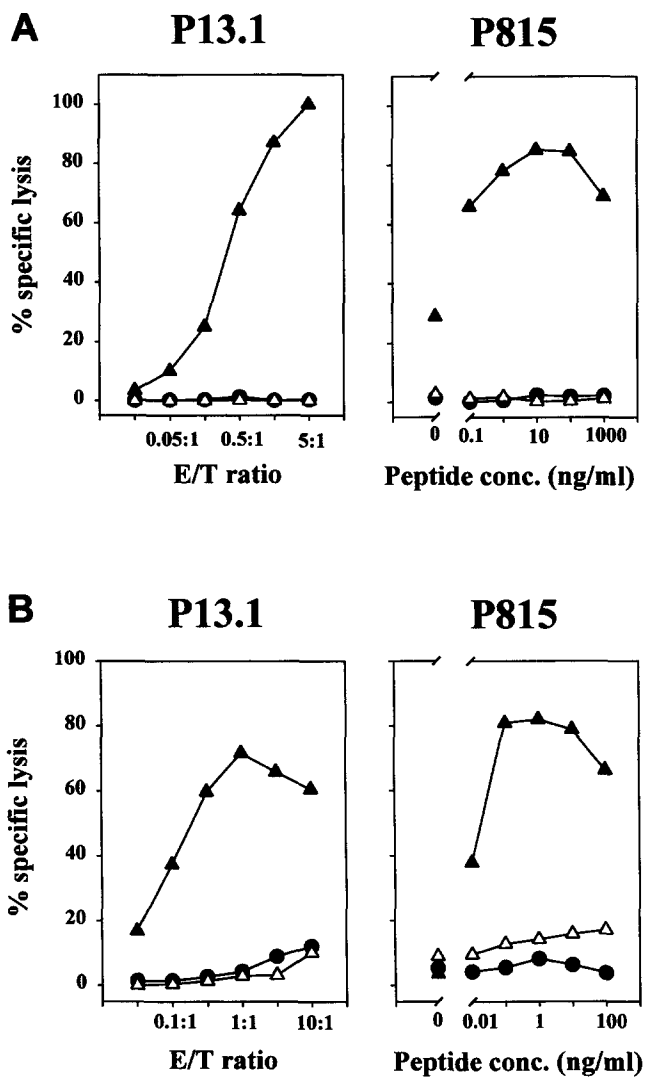

Figure 2. Induction of β-gal–specific CTL responses using gp96 molecules isolated from P815, P13.1, R/gal, and RS/gal cells. (*A*) BALB/c mice were immunized with gp96 molecules isolated from P815 cells (*open triangles*), P13.1 cells (*closed triangles*), or with PBS (*closed circles*). Spleen cells were stimulated in vitro with the β-gal–derived peptide TPHPARIGL and the CTL activity was tested on P13.1 cells at the indicated E/T ratio or on P815 incubated with different concentrations of the β-gal peptide at an E/T ratio of 3:1. (*B*) BALB/c mice were immunized with gp96 molecules isolated from RS/gal cells (*open triangles*), R/gal cells (*closed triangles*), or PBS (*closed circles*). Spleen cells were stimulated and CTL activity was determined as described in *A*.

not RS/gal cells is able to induce CTL recognizing P13.1 or β-gal peptide-incubated P815 cells. This experiment clearly shows that β-gal–derived peptides are loaded onto gp96 molecules inside the ER, as this process is not observed in cells with a TAP defect. These results were reproduced in three independent experiments with a gp96 concentration (30 μg), which is in threefold excess of the amount required for optimal tumor protection (2) and CTL induction, as determined by titrations of gp96 over a range from 2 to 30 μg (data not shown).

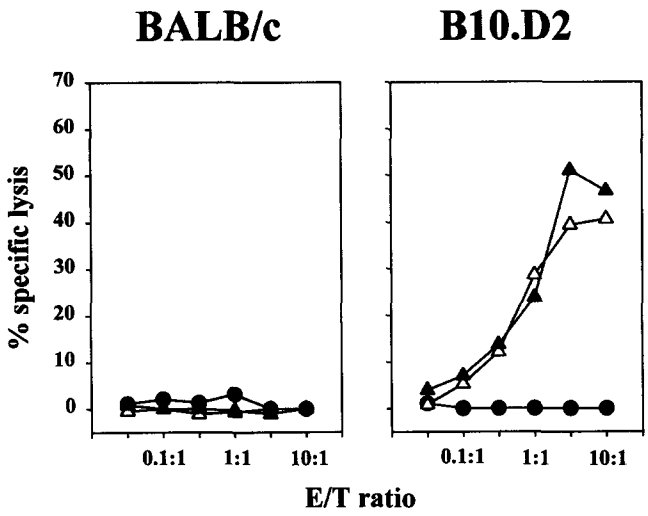

Figure 3. Induction of minor H-specific CTL using gp96 molecules isolated from R/gal and RS/gal cells in BALB/c mice. Mice were immunized with PBS (*closed circles*) or gp96 molecules isolated from RS/gal (*open triangles*), R/gal (*closed triangles*) to induce cross-priming against minor H antigens of the C57BL/6 background. Spleen cells were stimulated in vitro with B10.D2 stimulator cells and tested for their ability to lyse BALB/c (*left*) and B10.D2 (*right*) Con A blasts.

Generation of Minor H-specific CTL with gp96 from Cells Expressing Functional or Nonfunctional TAP Molecules. In the following experiments, we analyzed whether the TAP-dependent peptide loading of gp96 molecules is a general phenomenon that can be observed for other antigens as well. Therefore, BALB/c mice (H-2^d) were immunized with gp96 molecules from R/gal and RS/gal cells to induce cross-priming against minor H antigens expressed in C57BL/6 mice (H-2^b), from which R/gal and RS/gal cells were generated. The spleen cells from the immunized mice were stimulated with B10.D2 splenocytes (H-2^d), sharing most of the minor H antigens with C57BL/6. As shown in Fig. 3, spleen cells from the untreated mice showed no CTL activity, because the induction of minor H-specific CTL requires in vivo priming (15), but spleen cells from mice immunized with gp96 preparations from both R/gal and RS/gal displayed CTL activity against B10.D2 Con A blasts. A contamination of gp96 molecules from RS/gal cells with those from R/gal cells can be excluded because in some experiments spleen cells from mice immunized with RS/gal gp96 were also stimulated with β-gal peptides and never showed β-gal–specific CTL activity. The results could also be reproduced with gp96 molecules from untransfected RMA and RMA-S cells (data not shown). Therefore, these experiments show that the loading of peptides, reflecting some of the minor H antigens that differ between BALB and C57BL mice, onto gp96 molecules is independent of TAP. The origin of these minor H antigens is not known, but obviously their peptides enter the ER and are loaded onto gp96 molecules by a TAP-independent mechanism. This is not a rare phenomenon because some minor H antigens can be also recognized by H-2^b–restricted CTL in a TAP-independent fashion, as shown earlier (14) and

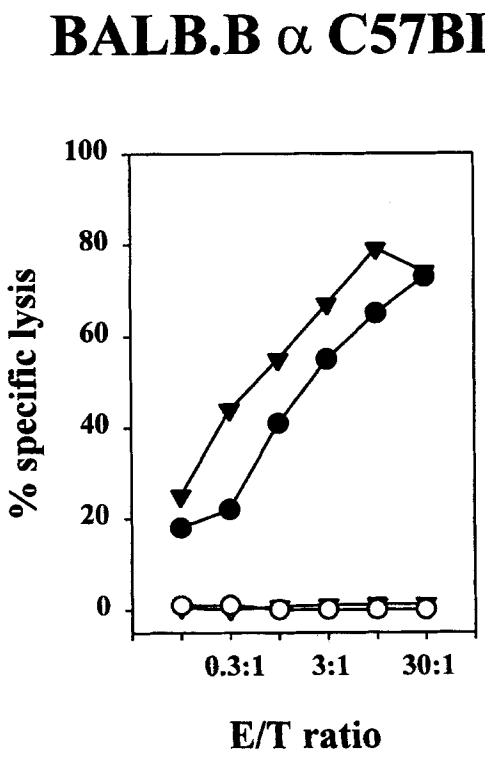

Figure 4. TAP-independent recognition of minor H antigens by BALB.B anti-C57BL/6 CTL. BALB.B mice were immunized with C57BL/6 spleen cells (*closed symbols*) or left untreated (*open symbols*) and stimulated in vitro with irradiated C57BL/6 cells. CTLs were tested for their ability to recognize RMA cells (*triangles*) or RMA-S cells (*circles*).

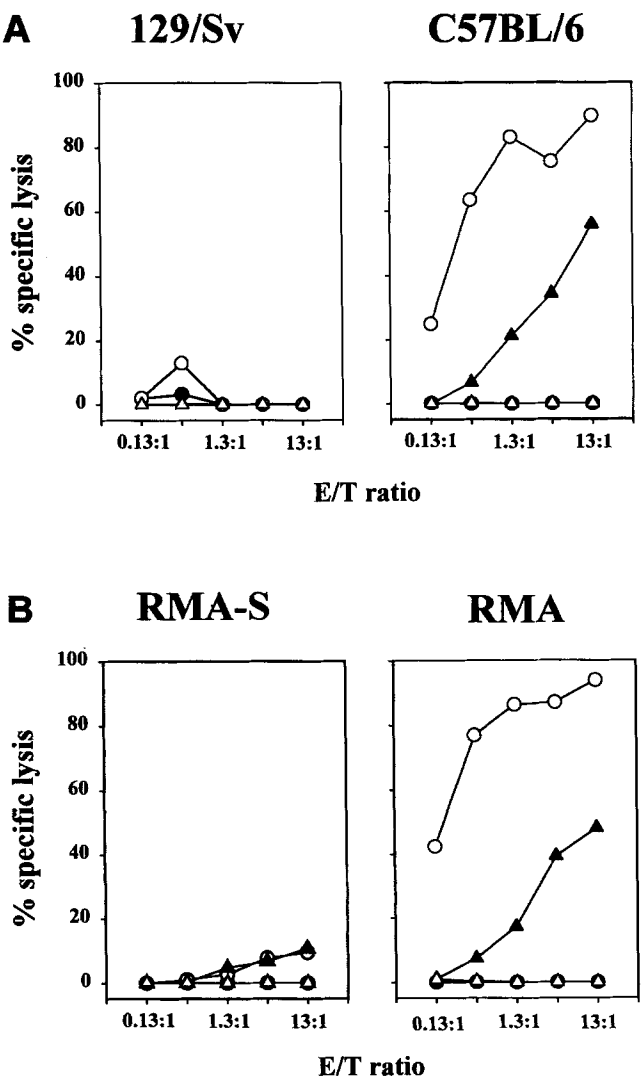

Figure 5. Induction of minor H-specific CTL in 129/Sv mice upon immunization with gp96 molecules isolated from RMA and RMA-S cells. Mice were immunized with gp96 molecules isolated from RMA-S cells (*open triangles*), RMA cells (*closed triangles*), C57BL/6 spleen cells (*open circles*) or with PBS (*closed circles*) to induce priming against minor H antigens of the C57BL/6 strain. Spleen cells were stimulated in vitro with C57BL/6 stimulator cells and tested for their ability to lyse 129/Sv and C57BL/6 Con A blasts (*A*) or RMA and RMA-S target cells (*B*).

again in Fig. 4. RMA and RMA-S cells are lysed comparably by CTL specific for minor H antigens differing between BALB.B and C57BL/6 mice, now presented by MHC molecules of the H-2^b haplotype. TAP-independent loading of peptides onto gp96 and MHC class I molecules is not observed in all minor H incompatible strain combinations. When we immunized 129/Sv mice with gp96 molecules from RMA or RMA-S cells to induce a CTL response against minor H-antigens that differ between 129/Sv and C57BL/6 mice, we observed that only gp96 molecules from RMA but not RMA-S cells were able to generate a minor H-specific CTL response (Fig. 5 *A*). This is in contrast with the experiments shown in Fig. 3 where the identical RMA-S gp96 preparation induced a CTL response in BALB/c mice against B10.D2 Con A blasts. In line with the above results, only RMA but not RMA-S cells are recognized by the minor H-specific CTL induced by immunization of 129/Sv mice with C57BL/6 cells or RMA-derived gp96 molecules (Fig. 5 *B*). This supports the notion that peptide loading onto MHC and gp96 molecules happens in the same intracellular compartment.

Discussion

Our results clearly show that the loading of gp96 molecules with T cell–recognized antigens is TAP dependent. Thus, gp96 must associate with peptide ligands inside the ER and its ability to induce specific CTL responses against antigens expressed in the cells from which it has been purified is not an artifact due to cell lysis during gp96 purification. This is evident from the immunization experiments with gp96 molecules isolated from cells with or without a defect in the TAP2 molecules and expressing a defined antigen, β-gal, exclusively in the cytosol. Only gp96 molecules isolated from TAP-competent cells induced a specific CTL response, indicating the association of β-gal–derived peptides with gp96 (Fig. 2). The same was observed for the induction of a CTL response against C57BL/6 cells in 129/Sv mice. Gp96 from RMA but not from RMA-S cells was

able to immunize against C57BL/6 minor H antigens (Fig. 5 *A*). This effect is not due to variations in the quality of the gp96 preparations from the different cell lines because identical gp96 preparations from RS/gal or RMA-S cells that failed to induce TAP-dependent β-gal or 129/Sv anti-C57BL/6 minor H-specific CTL responses were able to induce TAP-independent BALB/c anti C57BL/6 minor H-specific CTL (Fig. 3). In some cases, this was observed in CTL cultures derived from the same mouse (Fig. 2 *B*; Fig. 3). We also observed that CTL induced by immunization with C57BL/6 spleen cells or RMA-derived gp96 molecules recognized RMA but not RMA-S target cells (Fig. 5 *B*). This shows that not only the gp96 but also the MHC molecules of RMA-S cells completely lack those minor H antigen-derived peptides that are recognized by 129/Sv anti C57BL/6 CTL. These experiments, revealing a TAP-dependent loading of peptides onto gp96 molecules, are complemented by results obtained in vitro where radiolabeled peptides were added to streptolysin-O-permeabilized cells. In this study, it was shown that peptides associated with gp96 molecules only when the cells expressed functional TAP molecules and ATP was present (7, 16).

On the other hand, not all peptides associated with gp96 molecules depend on the presence of functional TAP molecules. This is evident from the ability of both RMA and RMA-S gp96 preparations to induce cross-priming against B10.D2 cells in BALB/c mice (Fig. 3). For at least a subset of these H-2^d–restricted peptides, the association with gp96 molecules is TAP independent. Interestingly, the presentation of H-2^b–restricted peptides in a similar combination of minor H antigens was also found to be TAP-independent: BALB.B-derived CTL specific for C57BL/6 cells recognize RMA and RMA-S cells to a similar extent (Fig. 4). RMA-S cells have been previously reported to present other peptide antigens including minor H antigens despite their mutation in the *TAP2* gene (14, 17). This presentation seems to be due to luminal production or to unknown intrinsic properties of these peptides, allowing either TAP-independent or just TAP1-mediated transport (16).

The above experiments indicate that gp96 does not exclusively receive its peptides from TAP molecules but also binds peptides that are present inside the ER due to TAP-independent translocation or that are generated inside the ER directly, possibly from ER-resident proteins. Our experiments might also provide an answer to the question how MHC molecules are loaded with peptides in TAP-deficient cell lines. In this situation, a direct loading of MHC molecules via their association with TAP (8, 18), or mediated by tapasin in human cells (19), is not possible. It seems feasible that gp96 or other ER-resident peptide-binding proteins like a 60-kD protein identified by Lammert et al. (7) or Marusina et al. (16), could be involved in these events. This might also include MHC class I loading of those peptides that require further processing before their binding to MHC molecules (20) and the removal of peptides from the ER that do not posses the constraints imposed by the ligand motif of the MHC molecules present in the ER. These functions were proposed for BiP (21) based on its ability to bind peptides in vitro (22). But it was clearly shown recently that BiP is not able to bind peptides translocated by TAP in streptolysin-O-permeabelized cells (7, 16). Therefore, new candidates, like gp96 or the 60-kD protein, are required for these services.

Even though the physiological role of gp96 is not defined yet, our results show that its peptide repertoire seems to reflect all the peptides that have entered the ER, either by TAP-dependent or -independent mechanisms. Combined with its ability to induce protective immune responses in vivo against a wide variety of antigens, including tumor antigens that are not further identified, our results provide the theoretical foundation for applying gp96 for the induction of immune responses against all peptides present in the ER, including those that are able to associate with MHC molecules.

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