Brief Definitive Report

Cross-priming of Minor Histocompatibility Antigen-specific Cytotoxic T Cells upon Immunization with the Heat Shock Protein gp96

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Summary

Vaccination of mice with heat shock proteins isolated from tumor cells induces immunity to subsequent challenge with those tumor cells the heat shock protein was isolated from but not with other tumor cells (Udono, H., and P. K. Srivastava. 1994. *J. Immunol.* 152:5398–5403). The specificity of this immune response is caused by tumor-derived peptides bound to the heat shock proteins (Udono., H., and P. K. Srivastava. 1993. *J. Exp. Med.* 178:1391–1396). Our experiments show that a single immunization with the heat shock protein gp96 isolated from β-galactosidase (β-gal) expressing P815 cells (of DBA/2 origin) induces cytotoxic T lymphocytes (CTLs) specific for β-gal, in addition to minor H antigens expressed by these cells. CTLs can be induced in mice that are major histocompatibility complex (MHC) identical to the gp96 donor cells (H-2^d) as well as in mice with a different MHC (H-2^b). Thus, gp96 is able to induce "cross priming" (Matzinger, P., and M. J. Bevan. 1977. *Cell. Immunol.* 33:92–100), indicating that gp96-associated peptides are not limited to the MHC class I ligands of the gp96 donor cell. Our data confirm the notion that samples of all cellular antigens presentable by MHC class I molecules are represented by peptides associated with gp96 molecules of that cell, even if the fitting MHC molecule is not expressed. In addition, we extend previous reports on the in vivo immunogenicity of peptides associated gp96 molecules to two new groups of antigens, minor H antigens, and proteins expressed in the cytosol.

The analysis of tumor rejection antigens has led to the identification of heat shock proteins being able to induce tumor-specific immunity in mice (1). Since heat shock proteins of tumor cells and normal tissue have been found to be of identical sequences, their tumor-specific immunogenicity was not easily understood. Recent experiments have provided an explanation to this paradox. Srivastava and colleagues observed that heat shock proteins (HSPs) chaperone antigenic peptides able to induce specific immune responses (1), although the detailed mechanisms leading to CTL activation after immunization with HSPs are not completely understood. Apparently, antigen processing and presentation by macrophages plays an important role (4). Among the HSPs identified to induce tumor specific immunity are hsp70, hsp90, and gp96 (1). Based on the observed association of CTL antigens with HSPs and supported by their intracellular location, Srivastava and co-workers (5) proposed a model where cytosolic- and endoplasmic reticulum (ER)-resident HSPs form a relay line chaperoning antigenic peptides from the cytosol into the ER and, after transport through the ER membrane, are involved in the transfer of peptides onto MHC class I

molecules. According to our own previous hypothesis on antigen processing (6, 7), the peptides should be gradually trimmed by cytosolic and ER-resident proteases during this transport. For some of the HSPs, like gp96, ATPase and proteolytic activities have indeed been reported (8). Since HSPs are nonpolymorphic proteins, the repertoire of bound peptides should reflect the repertoire of proteins inside the cell and should not be influenced by the MHC expression of the cell that the HSPs are isolated from. It should be possible, therefore, to induce a specific CTL response in mice of any given MHC type with HSPs derived from cells of the same or different MHC expression. The latter phenomenon is described as "cross-priming" (3), and it would suggest that HSPs play a role in this process, as proposed by Srivastava et al. (5). To test this model, we investigated the capacity of the HSP gp96 to induce a specific CTL response against a protein that is expressed only in the cytosol of the gp96 donor cells. In addition, we tested minor H-specific CTL responses after cross-priming of mice with a different MHC type than the gp96 donor cells. Our results support and extend the above-mentioned hypothesis.

Materials and Methods

Mice and Antibodies. BALB/c, DBA/2, BALB.B, 129/Sv, and C57BL/6 mice were obtained from Charles River Wiga (Sulzfeld, Germany), and they were maintained in the animal facilities at the German Cancer Research Center. Antibodies to gp96 (anti-grp94, SPA-850, clone 9G10) were obtained from StressGen Biotechnologies Corp. (Victoria, Canada).

Purification of gp96. gp96 was purified as described (1). Briefly, P13.1 cells 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 centrifuged at 100,000 *g.* The supernatant was applied to a Con A–Sepharose column and Con A–bound material was 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 by FPLC using a MonoQ column (5/5; Pharmacia Biotech AB, Uppsala, Sweden) with a 0–1 M NaCl gradient. Fractions were tested in SDS-PAGE and Western blot using an mAb specific for gp96. Fractions containing gp96 as the major protein were used for immunizations. The approximate concentration was determined by measuring the OD at 280 nm (extinction coefficient = 1.0).

Cell Culture and CTL Assays. P815 and P13.1 (9) cells were cultured in RPMI 1640 containing 10% FCS, 2-ME, L-glutamine, and antibiotics. CTL were generated by intraperitoneal immunization of mice with either 10^7 irradiated P13.1 cells (200 Gy) in 300 μl PBS or with 30 μg gp96 in 300 μl PBS. 10 d later, recipient spleens were removed, and the splenocytes were stimulated with minor H–incompatible, irradiated spleen cells (33 Gy) or with the synthetic β-gal peptide TPHPARIGL at 1 μM (10). CTL lines were generated by weekly restimulations with minor H–incompatible, irradiated spleen cells or with syngeneic, irradiated spleen cells and synthetic peptides as described (11). CTL assays were performed as described (11) using P815, P13.1, and Con A blasts prepared spleen cells as targets. Con A blasts were generated as described (12).

FACS® Analysis. 5 × 10^5 BALB.B-specific CTL, generated and maintained as described above, were incubated with a 100-fold dilution of FITC-conjugated anti-CD4, anti-CD8, or IgG control antibodies (Becton Dickinson & Co., Mountain View, CA) for 30 min on ice, washed three times with PBS/2% FCS, and analyzed on FACSort® (Becton Dickinson & Co.).

Results

Induction of β-gal–specific CTL. To test the hypothesis proposed by Srivastava and colleagues that gp96 binds peptides of cytosolic origin after they had been transported into the ER, we attempted to induce a β-gal–specific CTL response after intraperitoneal immunization of BALB/c mice (H-2^d) with gp96 molecules purified from the cell line P13.1 (H-2^d). This cell line is a transfectant of P815 that expresses *Escherichia coli*–derived β-gal from a construct without a leader sequence allowing only cytosolic expression (9, 13). Induction of β-gal–specific CTLs with gp96, therefore, should only be possible if β-gal–derived peptides are transported from the cytosol into the ER and subsequently bind to gp96 molecules. The purification of gp96 was done according to existing protocols, and the purity of the preparation was controlled by SDS-PAGE and Western blot analysis. As shown in Fig. 1, the gp96 material used for immunizations gives a single band in an SDS-PAGE and Western blot. Spleen cells
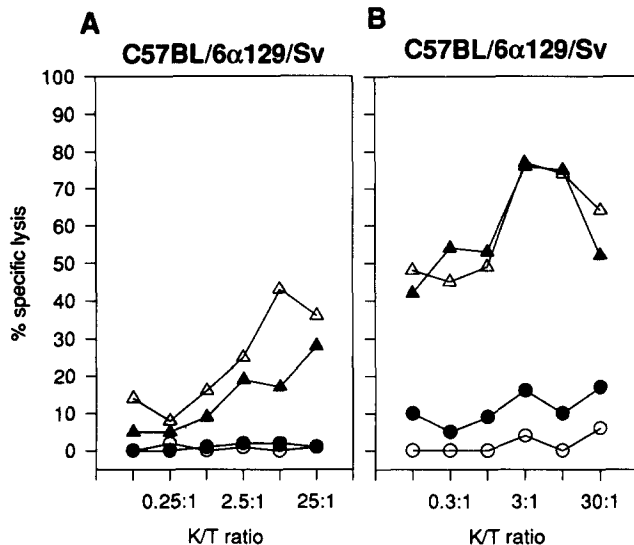

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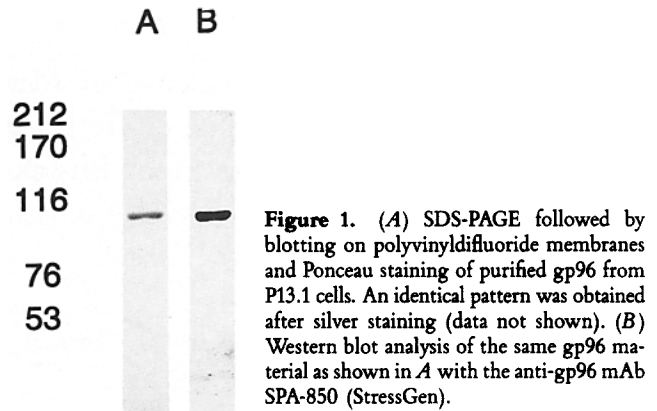
Figure 1. (*A*) SDS-PAGE followed by blotting on polyvinyldifluoride membranes and Ponceau staining of purified gp96 from P13.1 cells. An identical pattern was obtained after silver staining (data not shown). (*B*) Western blot analysis of the same gp96 material as shown in *A* with the anti-gp96 mAb SPA-850 (StressGen).

of immunized and untreated control mice were stimulated in vitro with the dominant H-2^d–restricted β-gal T cell epitope, the H-$2L^d$–restricted peptide TPHPARIGL (10). Specific CTL activity after 5 d of bulk culture was usually <20% on P13.1 target cells (data not shown). Therefore, CTL were restimulated with the β-gal–derived peptide for an additional 5 d and tested again on P815 and P13.1 target cells. Significant β-gal–specific CTL activity on P13.1 target cells was observed in cultures obtained from mice immunized with P13.1–derived gp96, whereas spleen cells from untreated mice showed no CTL activity (Fig. 2 *A*). Thus, gp96 molecules from P13.1 cells are able to prime β-gal–specific CTLs in mice, and therefore must have been carrying β-gal–derived material.
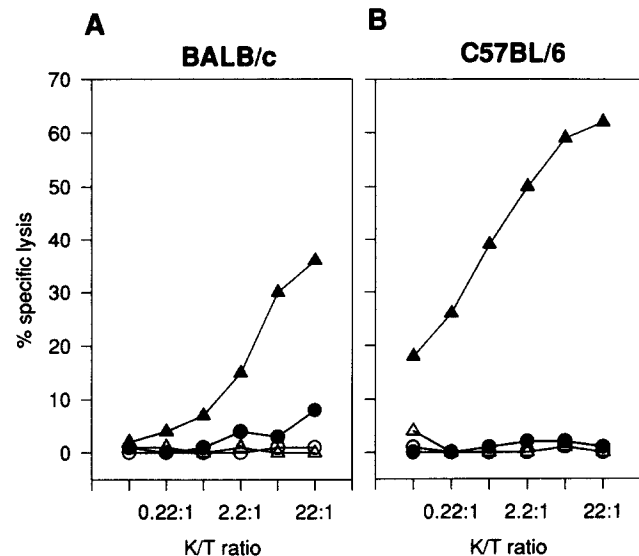
Figure 2. CTL activity of mice after immunization with gp96 molecules. (*A*) Untreated (*open symbols*) or P13.1- derived gp96 immunized DBA/2 mice (*closed symbols*) were stimulated twice in vitro with β-gal–derived peptides, and CTL were tested on P815 (*circles*) or P13.1 (*triangles*) target cells. Spontaneous ^{51}Cr release was <10%, and specific lysis was calculated as described (11). (*B*) Untreated (*open symbols*) or P13.1-derived gp96 immunized C57BL/6 mice (*closed symbols*) were stimulated twice in vitro with BALB.B spleen cells, and CTL were tested on C57BL/6 (*circles*) and BALB.B (*triangles*) Con A blasts as target cells. Spontaneous release was <18%.

Cross-priming of Minor H–specific CTLs. To investigate if gp96 chaperones antigenic peptides independently of the MHC coexpressed in the cell and is therefore able to induce CTL responses in mice that do not match the MHC type of the gp96 donor cells, we also immunized C57BL/6 mice (H-2^b) with gp96 isolated from P13.1 cells (H-2^d). 10 d after immunization, recipient splenocytes were stimulated with irradiated BALB.B spleen cells. P13.1 is of DBA/2 (H-2^d) origin. DBA/2 differs from C57BL/6 mice at multiple minor H genes. Many of the DBA/2 minor H antigens are also expressed by the strains BALB.B and 129/Sv (both H-2^b), including antigens encoded in the H-3 and H-4 regions. Thus, immunization of C57BL/6 mice with DBA/2-derived cells should prime BALB.B and 129/Sv-reactive CTL in the recipients, as shown below. Similarly, DBA/2-derived gp96 molecules should also prime C57BL/6 mice against BALB.B and 129/Sv minor H antigens. As shown in Fig. 2 *B*, P13.1 (H-2^d)-derived gp96 was indeed able to generate CTLs that recognize minor H antigens expressed on BALB.B (H-2^b) but not on C57BL/6 (H-2^b) Con A blasts. Unimmunized C57BL/6 mice did not generate a response, as expected, since minor H–specific CTL responses require in vivo priming (14). To compare the immunogenicity of the gp96 preparation with that of intact P13.1 cells, B6 mice were immunized with either P13.1 cells or P13.1-derived gp96. After 10 d, splenocytes were stimulated with 129/Sv stimulator cells and the CTL activity was tested after 5 d of culture and after two rounds of restimulation (Fig. 3, *A* and *B*). As shown in Fig. 3 *A*, a slightly stronger CTL response was observed after immunization with P13.1 cells as compared to immunization with gp96. After restimulation, however, this difference
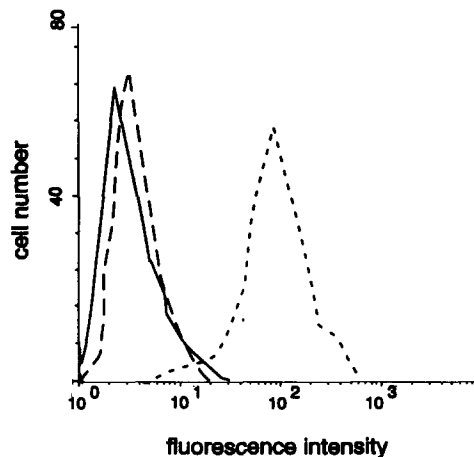
could not be detected any more, as shown in Fig. 3 *B*, since both cultures lysed 129/Sv blasts equally well. Again, spleen cells from mice that were not immunized showed no CTL activity, and after several rounds of restimulation, no growing cells were detectable anymore (data not shown). The minor H–specific CTL lines induced by cross-priming with gp96 molecules are CD8 positive, as shown for the BALB.B-specific CTL line in Fig. 4.

Our experiments show that immunizations with gp96 molecules induce CD8-positive T cell responses that are roughly as effective as immunizations with intact tumor cells. Spleen cells, however, which are usually more immunogenic than tumor cells, might be expected to be more effective than gp96.

Discussion

Firstly, our experiments confirm that the HSP gp96 is complexed with antigens (15) that are able to induce a specific CTL response after intraperitoneal immunization without the use of additional adjuvants. These antigens (probably peptides) are derived from cellular proteins; in our case, from β-gal or from undefined self-proteins representing minor H antigens. Secondly, our results demonstrate that the ER-resident HSP gp96 carries antigens of cytosolic origin, since P13.1 expresses the β-gal gene without a leader sequence (9), and gp96 derived from these cells primes β-gal–specific CTLs in mice. Thirdly, the induction of minor H–specific CTLs with gp96 derived from an H-2^d cell in H-2^b mice demonstrates the cross-priming capability of gp96 molecules.

Since the same gp96 preparation can induce CTL specific for different antigens, many different peptides have to be bound, most likely in different amounts. This might explain the need for additional in vitro restimulations for certain antigens, as observed for β-gal–specific CTLs. In addition to minor H antigens and β-gal, viral antigens and tumor antigens have been shown to be associated with gp96 and to induce a CTL response upon immunization (15).

Figure 3. Comparison of the CTL activity of C57BL/6 mice after immunization with P13.1 cells or P13.1-derived gp96 molecules after 5 d of in vitro stimulation (*A*) and after two rounds of weekly in vitro restimulations (*B*) with 129/Sv spleen cells. Mice were immunized with P13.1 cells (*open*) or gp96 molecules (*closed*) and stimulated for 5 d with 129/Sv spleen cells. CTL activity was tested on C57BL/6 (*circles*) and 129/Sv (*triangles*) Con A blasts. Spontaneous ^{51}Cr release was <20%.

Figure 4. FACS® analysis of minor H–specific CTL induced by immunization with gp96. A BALB.B specific CTL line was stained with FITC-conjugated anti-CD4 (*solid line*), anti-CD8 (*dotted line*) antibodies, and control IgG (*dashed line*).

Our results support the hypothesis that gp96 is part of the machinery that is responsible for protein degradation and peptide loading onto MHC class I molecules. According to this hypothesis, gp96 molecules bind peptides of cytosolic origin after they have been transported through the ER membrane in a process involving the transporter associated with antigen processing (TAP) molecules. These peptides, being the product of the cytosolic part of antigen processing (probably involving the proteasome complex), are transported in an ATP-dependent manner from gp96 onto MHC class I molecules (5). Since no peptides of the correct length required for MHC class I presentation have been isolated from total cell extracts of MHC-mismatched cells (6) and since the peptide transporter seems to transport peptides consisting of 9–16 amino acids (16), we believe that gp96-bound peptides are intermediates of antigen processing that require further trimming inside the ER (7) until they can be presented by MHC class I molecules. In addition to peptides transported by the TAP molecules, gp96 might also be able to bind peptides that enter the ER in a TAP- independent manner or that are generated inside the ER. The functions of gp96 during the final peptide-trimming events are not known, but the suggested proteolytic activities of gp96 and other HSPs, including ATPase activity (8), allow the speculation that gp96 itself could play an active role in the ER-resident processing of MHC class I ligands. One possibility is that gp96 generates the correct COOH-termini required for the precursor peptides to bind to the COOH-terminal pocket of MHC class I molecules. The processed peptides would then be transferred onto MHC molecules where the final NH_2-terminal trimming steps occur, generating peptides of the right size to fit into the protecting binding groove, as previously suggested (6). The frequent use of hydrophobic COOH-terminal anchor residues observed among ligands of many MHC class I molecules might therefore also reflect the specificity of members of the processing machinery and not solely selection by MHC molecules, as has been suggested earlier (17). The possibility of gp96 being involved in antigen processing is currently under investigation in our laboratory. Alternatively, the role of gp96 might just be the binding of transported precursor peptides, and by doing this, allowing NH_2- and/or COOH-terminal trimming by ER-resident proteases, but at the same time also protecting peptides from complete degradation. The MHC class I molecules then might select from the pool of gp96-bound precursor peptides, those containing the corresponding ligand motifs that allow, after the generation of the correct NH_2- and COOH-termini, binding to the MHC class I molecules.

Both models, suggesting either an active role in the trimming of peptides or a more passive role as a peptide carrier, explain the ability of gp96 to cross-prime for minor H antigens since they predict the binding of precursor peptides independent of the MHC molecules coexpressed in the cell.

In agreement with the proposed function of gp96 in antigen processing and presentation, it has been noted that the expression of gp96 is upregulated by IFN (18), as reported for other molecules of the antigen presentation pathway, such as proteins of the proteasome complex (19), the peptide transporters (20), and MHC molecules themselves (21). The capability of gp96 to induce CTL responses across MHC barriers without the use of adjuvants underlines its promise as a vaccine (1, 2, 4, 5, 8). A possible application could be the immunization with gp96 molecules isolated from tumor cells, expressing nonprivate antigens (22) or cells infected with viruses or intracellular parasites known to induce specific and protective CTL responses.

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