Minor Histocompatibility Antigen-Specific MHC-Restricted CD8 T Cell Responses Elicited by Heat Shock Proteins

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In mammals, the heat shock proteins (HSP) gp96 and hsp70 elicit potent specific MHC class I-restricted CD8⁺ T cell (CTL) response to exogenous peptides they chaperone. We show in this study that in the adult frog *Xenopus*, a species whose common ancestors with mammals date back 300 million years, both hsp70 and gp96 generate an adaptive specific cellular immune response against chaperoned minor histocompatibility antigenic peptides that effects an accelerated rejection of minor histocompatibility-locus disparate skin grafts in vivo and an MHC-specific CD8⁺ cytotoxic T cell response in vitro. In naturally class I-deficient but immunocompetent *Xenopus* larvae, gp96 also generates an antitumor immune response that is independent of chaperoned peptides (i.e., gp96 purified from normal tissue also generates a significant antitumor response); this suggests a prominent contribution of an innate type of response in the absence of MHC class I Ags. Given the high degree of structural conservation of HSPs among vertebrates and invertebrates, it has been proposed that some of their immunological properties may also have been conserved during evolution. Indeed, we have shown that the *Xenopus* gp96 protein homolog stably and noncovalently complexes peptides in vitro, and that peptides chaperoned by *Xenopus* gp96 can be efficiently represented by MHC class I of mouse macrophages to specifically stimulate murine class I-restricted CTL lines. Moreover, immunization of *Xenopus* with gp96 purified from a highly tumorigenic class I-negative *Xenopus* thymic tumor generates potent antitumor immunity as measured by a significant delay in the appearance and diminished size of tumors after challenge. No such effect has been observed with gp96 from normal tissues. Similar antitumor responses result from priming with a purified *Xenopus* gp9670-tumor peptide complex, but not with tumor-derived hsp70 that is free of peptide.

The immune system of the adult African clawed frog *Xenopus* is fundamentally similar to that of mammals (e.g., rearranging TCR and Ig genes, and MHC class I- and class II-restricted T cell recognition; reviewed in Refs. 21 and 23). However, MHC-restricted Ag-specific T cell clones or lines are not yet available in *Xenopus*, and the transplanted tumors we use do not express the MHC class I molecule (24). Therefore, to study the phylogenetic conservation of HSP T cell adjuvanticity properties in our *Xenopus* model, we assessed the ability of HSPs to generate an MHC-restricted CD8 T cell-mediated response against chaperoned exogenous antigenic peptides encoded for by *Xenopus* minor histocompatibility (H) genes. The involvement of MHC class I representation was further evaluated by studying tumor-derived gp96-induced immunity in naturally MHC class I-deficient larvae. We report in this study that both *Xenopus* gp96 and hsp70 generate specific in vivo cellular immune responses and in vitro CTL responses against chaperoned minor H-Ag peptides. Furthermore, in naturally MHC class I-deficient larvae, gp96 elicits a significant antitumor response even when it is purified from normal (non-tumor) tissue, suggesting it can evoke a non-MHC-restricted innate type of antitumor response. The finding that immunological properties of HSPs have been conserved since amphibian and mammalian lineages diverged some 300 million years ago provides, by itself, a strong “evolutionary” validation of their biological importance.

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3 Abbreviations used in this paper: HSP, heat shock protein; H, histocompatibility; OB, outbred; APBS, amphibian PBS; DC, dendritic cell.

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**Materials and Methods**

**Animals and tumor lines**

Outbred (OB) and inosogenous (cloned; Ref. 25) MHC heterozygous LG-15, LG-6, and LG-46 (MHC haplotype a/c) Xenopus were obtained from our breeding colony. The 150 lymphoid tumor line, derived from spontaneously arising thymic tumors of LG-15 animals, is MHC class I and class II negative (24, 26).

**Purification of Xenopus gp96 and hsp70**

Gp96 was purified as previously described (15) by 50–70% ammonium sulfate fractionation, concanavalin A-Sepharose, and DEAE chromatography. hsp70 was purified as previously described (27) by Blue-Sepharose chromatography to remove albumin contaminants and either an ADP-agarose or ATP-agarose column (Sigma-Aldrich, St. Louis, MO), followed by DEAE chromatography. Purity was assessed by SDS-PAGE and silver staining, and by Western blotting (data not shown).

**Flow cytometry**

Cells (10^6 cells) were stained with undiluted hybridoma supernatants specific for Xenopus CD8 (AM22, Ref. 28; F17, Ref. 29), CD5 (2B1; Ref. 30), pan T cell (XT-1; Ref. 31), IgM (10A9; Ref. 32), or isotype-matched mAb controls followed by fluorescein-labeled goat anti-mouse Ig F(ab')2 that had been preadsorbed twice on Xenopus erythrocytes. The stained cells were then analyzed by flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA), 10,000 events were collected, and dead cells stained by propidium iodide were gated out.

**Immunization and skin grafting**

LG-6 isogenic Xenopus were injected two or three times (2-wk interval) with either irradiated (50 Gy) 15/0 tumor (LG-15-derived), LG-15 total proteins (100,000 × g supernatant from Xenopus liver), or with purified gp96 in 0.3 ml of amphibian PBS (APBS). Two weeks after the last immunization, LG-6 animals were gated according to published methods (33). Briefly, a piece of ventral skin (5 mm²) was inserted under the dorsal skin of a recipient, and 48 h later the overlying host skin was removed. The onset of rejection was marked by initiation of pigment cell death, and rejection was considered complete when all pigment cells in the graft were destroyed.

**In vitro killing assay**

Splenocytes from naive or immunized frogs were harvested and restimulated (5 × 10^6 cells/ml) in vitro for 6 days with one-half as many irradiated LG-15 splenocytes in medium containing 0.25% Xenopus propidium iodide were gated out. Flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA), 10,000 events were collected, and dead cells stained by propidium iodide were gated out.

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**In vitro killing assay**

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**Larval tumor assay**

Tadpoles were blocked at premetamorphic stage 55 with sodium perchlorate (1 g/l) in their aquarium water, and they were primed i.p. with two injections of one of the following: 5 μl of APBS; 10⁴, 10⁵, or 10⁶ irradiated tumor cells in 5 μl of culture medium; 5 μl of 15/0 whole protein lysate (60 ng each); or 5 μl of purified 15/0 gp96, or normal LG-15 (12 or 60 ng each). The second injection was given 2 wk after the first, and larvae were challenged i.p. with 5 × 10⁵ 15/0 tumor cells (<5% cell death) 2 wk after the second injection. On day 14 after challenge, peritoneal fluid from each tadpole was collected and the tumor cells were counted. Host survival after tumor challenge was monitored daily.

**Results**

**Generation of anti-minor H Ag responses in vivo by gp96 and hsp70**

Thymus dependency and Ag specificity of skin allograft rejection are well characterized in Xenopus thanks to MHC-defined inbred strains and isogenic clones (33, 36–40). LG-15, LG-6, and LG-46 isogenic Xenopus clones all display the identical heterozygous (a/c) MHC genotype (25). However, these clones differ from each other by multiple minor H loci (24, 40–42). Skin grafts transplanted between these clones are rejected significantly more slowly (30–50 days at ~21°C; see Fig. 1) than are MHC disparate allografts (20 ± 2 days at ~21°C). Gp96 purified from LG-15, LG-46, or LG-6 liver was used to immunize LG-6 adult animals. Immunization with 10 μg of LG-15-derived gp96 twice (a 2-wk interval) significantly and consistently accelerated rejection of LG-15 skin grafts relative to control LG-6 injected with either APBS, total LG-15 protein lysate, or autologous LG-6-derived gp96 (Fig. 1, A–C). Immunized animals did not reject autologous LG-6 skin (survival, >100 days), whereas they did reject MHC-disparate LG-5 (b/c haplotype) skin in 20 ± 2 days (data not shown). Similar accelerated rejection of LG-46 skin was obtained.
by immunizing LG-6 recipients with LG-46-derived gp96 (Fig. 1D). In this experiment, LG-6 animals were simultaneously grafted with both LG-46 and LG-15 skin. Only rejection of LG-46 skin was accelerated in immunized animals, whereas the mean rejection times (± SD) for LG-15 grafts were comparable between control and immunized groups (45.4 ± 3.5 and 47 ± 2.8, respectively).

To better determine the need for chaperoned peptides in the generation of a specific anti-minor H-Ag response, we compared the immunogenicity of hsp70-peptide complexes (purified by ADP-agarose chromatography) with hsp70 without peptide (purified by ATP-agarose chromatography; Refs. 14 and 22). Immunization with LG-15-derived hsp70-peptide complexes induced an accelerated rejection comparable with LG-15-derived gp96, whereas hsp70 without peptide had no effect on skin graft survival (Fig. 1E).

**Effect of HSP immunization on cell-mediated cytotoxicity**

Although thymus-dependent, MHC-specific cytotoxic activity against both major (43, 44) and minor (45) H-Ags has been described in *Xenopus*, the involvement of MHC-restricted CD8 cytotoxic T cells in this alloreactivity has not been formally demonstrated. To examine whether *Xenopus* CD8 T cells are CTL, *Xenopus* CD8 T cells were separated from splenocytes using a *Xenopus*-specific anti-CD8 mAb (46) and magnetic microbeads (MACS); cytotoxic activity of the positively and negatively selected cells was then studied in vitro. As determined by flow cytometry, the MACS-sorted population contained >90% CD8+ cells (Fig. 2A). This population also stained positive for the pan-T cell marker, XT-1 (Ref. 30 and data not shown) and CD5 (T cell marker in *Xenopus*; Ref. 30) but was negative for surface IgM (Fig. 2A). The splenocytes remaining after selection for CD8+ cells (referred to as CD8-depleted) contained some CD8+ cells (10–20%), CD5+ T cells, and IgM+ B cells.

To determine the cytotoxic activity of *Xenopus* CD8+ T cells purified from alloimmunized frogs, splenocytes harvested from OB animals that had been immunized against LG-15 Ags by multiple LG-15 skin grafts were restimulated in vitro with irradiated LG-15 spleen cells (43). When tested for cytotoxic activity using the “just another method” technique (a more sensitive DNA fragmentation assay than the classical 51Cr-release assay; Ref. 34), these CD8+ T cells (determined by flow cytometry) showed a strong and specific cytotoxic activity against LG-15 splenocytes but not against MHC-disparate (non-a/c) PHA-lymphoblast targets. NK-like activity against MHC-negative tumor targets was minimal (Fig. 2, C and D). Similarly, CD8-selected T cell effectors from LG-6 animals immunized against LG-46 minor H-Ags by multiple skin grafts specifically killed LG-46 but not cognate LG-6 PHA-lymphoblast targets (Fig. 2, E and F). Specific killing activity against LG-15 lymphoblasts targets by CD8 purified T cells was also obtained by immunizing LG-6 recipients with multiple skin grafts from LG-15 minor H-Ag disparate donors; splenocytes from these LG-6 animals specifically killed LG-15 but not cognate LG-6 PHA-lymphoblast targets (data not shown).

Given this capacity of *Xenopus* to generate a MHC-restricted CD8-T cell response, we next investigated whether the immune response elicited against minor H-alloantigens by HSV-peptide complexes also involved CD8 cytotoxic T cells. CD8+ T cell effectors from LG-6 animals immunized with hsp70-peptide complexes purified from LG-15 tissue, but not control CD8- lymphocytes from LG-6 immunized with peptide-free hsp70 or from naive LG-6, showed specific reproducible killing against LG-15 blast targets (Fig. 3, A–C). The remaining CD8-depleted effectors showed reduced killing activity against minor H-Ag disparate class I+ targets, but a strong NK-like activity against class I-negative 15/0 tumor targets derived from LG-15 animals (Fig. 3, D–F).

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**FIGURE 2.** CTL characterization against major and minor-H Ags. A, Flow cytometry of CD8+ T cells positively selected with magnetic microbeads, 10,000 events (<5% IgM+ B cells, >90% are CD8+ and CD5+, which is a T cell marker in *Xenopus*). B, Phenotype of remaining cells after sorting (10–20% CD8+, 20% IgM+ B cells, and 30% CD5+). C, Cytotoxic activity of CD8-selected anti-LG-15 effectors from OB (MHC-disparate) frogs. D, Cytotoxic activity of CD8-depleted anti-LG-15 effectors from OB frogs. E, Cytotoxic activity of CD8-selected LG-6 anti-LG-46 (minor H-disparate) effectors. F, Cytotoxic activity of CD8-depleted LG-6 anti-LG-46 (minor H-disparate) effectors. ▲, OB PHA-lymphoblasts; ●, LG-6 PHA-lymphoblasts; ○, LG-15 PHA-lymphoblasts; □, LG-46 PHA-lymphoblasts; ●, class I-negative 15/0 tumor targets. Splenocytes from OB animals that had rejected MHC-disparate LG-15 (a/c) skin (C and D) and from LG-6 animals that had rejected minor H-Ag disparate LG-15 skin (E and F) were restimulated in vitro for 6 days with irradiated LG-15 or LG-46 spleen cells, respectively. CD8+ T cells were sorted with anti-CD8 AM22 mAb and anti-mouse IgM magnetic microbeads and were cultured overnight before being assayed for killing. 150 tumor (10,000) or PHA-blast targets (25,000) labeled with [3H]Tdr were incubated with various number of effectors, in triplicate, for 4 h at 26°C in 96-well plates. Thymidine uptake was determined by scintillation spectrometry. The percentage of killing activity was calculated by determining loss of apoptotic DNA relative to target alone control ([cpm elution — experimental]/[cpm control] × 100). Variation within triplicate groups was <10% of group mean cpn. The cpm value for 10,000 tumor targets ranged between 5,000 and 6,000 cpn, and the cpm value for 25,000 PHA-lymphoblasts ranged between 1,000 and 1,200 cpn.
of MHC class I presentation in the context of HSP immunogenicity. LG-15 larvae were blocked by sodium perchlorate at metamorphic stage 55; this delays class I expression for several weeks (50). Before challenge with 15/0 tumor cells, larvae were primed with APBS, irradiated 15/0 tumor cells, 15/0 total protein lysates, or different concentrations of tumor- or normal tissue-derived gp96. Growth of tumor in animals injected with irradiated 15/0 cells or with 15/0 whole protein lysate did not differ from tumor growth in APBS-injected controls (p > 0.05; Fig. 5, A and B). By contrast, injection of gp96 from either tumor or normal tissue resulted in significantly decreased tumor growth compared with controls. The partial tumor growth inhibition of tumor-derived and normal tissue-derived gp96 did not differ statistically (Student’s t test; p > 0.5). In addition to retarding tumor growth, normal- and tumor-derived gp96 were associated with prolonged host survival. Nonparametric statistical analysis (Mantel-Cox; StatView; Abacus Concepts, Berkeley, CA) indicates that the survival of tadpoles immunized with either tumor- or normal tissue-derived gp96 and subsequently challenged with viable tumor cells was significantly prolonged relative to nonimmunized control tadpoles (p = 0.001 and 0.027, respectively). However, the survival of tadpoles in these two experimental groups did not differ (p > 0.46).

Discussion

Cell-mediated cytotoxicity has been characterized in vitro in a few nonmammalian taxa, including teleosts (51) and Xenopus, a species where both NK-like (52, 53) and MHC-specific CTL-like (43, 45, 54) killing has been documented. Xenopus CD8 T cells have been characterized by two mAbs (known as F17 (29) and AM22 (28, 46)); each stains 85–90% of total thymocytes and 20–30% of splenic lymphocytes. The anti-CD8 mAb AM22 binds a polypeptide of 30–32 kDa as determined by Western blotting (28), and it immunoprecipitates a dimeric complex of 65 kDa under nonreducing conditions that resolves to a 35-kDa band and a 30- to 32-kDa band under reducing conditions (26). Xenopus CD8 T cells stained with either of the two anti-CD8 mAbs express a pan-T cell marker (XT-1), high levels of CD5, and CD45 (55–57); these cells are not detectable in animals that had been thymectomized at early developmental stages before the migration of stem cells into the thymus (55). Finally, in vitro studies suggest that the mammalian type of thymocyte differentiation (i.e., immature double-positive CD4CD8+ cells giving rise to more mature single positive CD4+CD8− and CD8+CD4−) also occurs in both MHC class I-negative larvae and class I-expressing adults (56, 57). The formal demonstration in Xenopus of an effector cell that is the phenotypic and functional equivalent of the mammalian CD8+ cytotoxic T cell was prerequisite to assessing whether the ability of some HSPs to function as natural T cell adjuvants is a fundamental property of HSP that has been maintained during evolution. Our data show for the first time in a cold-blooded vertebrate the existence of MHC-restricted cytotoxic effectors that are pan-T cell*, CD8*, CD5*, and IgM−. In mice, antigenic peptides chaperoned by hsp70 or gp96 elicit potent CTL responses against a large variety of chaperoned antigenic peptides, including those from tumors (1, 4, 9), viruses (10, 11), skin grafts (12), and model Ags (13, 14). The proposed mechanism by which such a robust response is generated involves internalization of the HSP-peptide complex by APCs after its interaction with a surface receptor, and the channeling of the peptides into the class I presentation pathway (1, 7). Recently, a surface receptor (CD91) expressed by DC and a subset of macrophages has been shown to bind gp96-peptide and lead to its rapid internalization (19). Receptor-mediated uptake of hsp70 by APC has also been documented (18). We have reported that Xenopus.

**Immunogenicity of gp96-peptide complexes in MHC class I-negative tadpoles**

Larval Xenopus are immunocompetent, but cell surface expression of MHC class I molecules does not occur in most tissues, including the thymus, until metamorphosis (47, 48). Furthermore, because LMP7 gene expression also does not occur until metamorphosis (49), MHC class I-restricted peptide presentation in tadpoles is likely to be absent or, at best, inefficient. We took advantage of this unique natural MHC deficiency to further explore the requirement of HSP presentation, as well as the nature of the bystander effect in Xenopus.

**Cell-mediated cytotoxicity has been characterized in vitro in a few nonmammalian taxa, including teleosts (51) and Xenopus, a species where both NK-like (52, 53) and MHC-specific CTL-like (43, 45, 54) killing has been documented. Xenopus CD8 T cells have been characterized by two mAbs (known as F17 (29) and AM22 (28, 46)); each stains 85–90% of total thymocytes and 20–30% of splenic lymphocytes. The anti-CD8 mAb AM22 binds a polypeptide of 30–32 kDa as determined by Western blotting (28), and it immunoprecipitates a dimeric complex of 65 kDa under nonreducing conditions that resolves to a 35-kDa band and a 30- to 32-kDa band under reducing conditions (26). Xenopus CD8 T cells stained with either of the two anti-CD8 mAbs express a pan-T cell marker (XT-1), high levels of CD5, and CD45 (55–57); these cells are not detectable in animals that had been thymectomized at early developmental stages before the migration of stem cells into the thymus (55). Finally, in vitro studies suggest that the mammalian type of thymocyte differentiation (i.e., immature double-positive CD4CD8+ cells giving rise to more mature single positive CD4+CD8− and CD8+CD4−) also occurs in both MHC class I-negative larvae and class I-expressing adults (56, 57). The formal demonstration in Xenopus of an effector cell that is the phenotypic and functional equivalent of the mammalian CD8+ cytotoxic T cell was prerequisite to assessing whether the ability of some HSPs to function as natural T cell adjuvants is a fundamental property of HSP that has been maintained during evolution. Our data show for the first time in a cold-blooded vertebrate the existence of MHC-restricted cytotoxic effectors that are pan-T cell*, CD8*, CD5*, and IgM−. In mice, antigenic peptides chaperoned by hsp70 or gp96 elicit potent CTL responses against a large variety of chaperoned antigenic peptides, including those from tumors (1, 4, 9), viruses (10, 11), skin grafts (12), and model Ags (13, 14). The proposed mechanism by which such a robust response is generated involves internalization of the HSP-peptide complex by APCs after its interaction with a surface receptor, and the channeling of the peptides into the class I presentation pathway (1, 7). Recently, a surface receptor (CD91) expressed by DC and a subset of macrophages has been shown to bind gp96-peptide and lead to its rapid internalization (19). Receptor-mediated uptake of hsp70 by APC has also been documented (18). We have reported that Xenopus.

**FIGURE 3.** Generation of anti-minor H-Ag-specific CTL by immunization with hsp70-peptide complexes. Splenocytes from naive (A and D), immunized LG-6 with 10 μg (three times s.c.) of liver LG-15-derived ATP-purified (peptide-depleted) hsp70 (B and E), or 10 μg of LG-15 liver-derived ADP-purified (peptide-loaded) hsp70 (C and F). LG-6 splenocytes were restimulated in vitro with irradiated LG-15 spleen cells. Killing activity of CD8 selected (upper line) and CD8-depleted (lower line) effectors was assayed against LG-15 PHA-lymphoblasts (○), LG-6 PHA-lymphoblasts (□), and class I-negative 15/0 tumor (▲) targets as described for Fig. 2.

**FIGURE 4.** Generation of anti-minor H-Ag-specific CTL by immunization with gp96-peptide complexes. A and D, Splenocytes from LG-6 animals immunized (three times s.c.) with 10 μg of LG-15 liver-derived gp96, and restimulated in vitro with irradiated LG-15 splenocytes. B and F, Splenocytes from LG-6 animals immunized (three times s.c.) with 10 μg of cognate LG-6 liver-derived gp96, and restimulated in vitro with irradiated LG-6 splenocytes. C and G, Splenocytes from LG-6 animals immunized (three times s.c.) with 10 μg of LG-46 liver-derived gp96 were restimulated in vitro with irradiated LG-46 cells. Killing activity of CD8+ selected (upper line) and the remaining CD8-depleted (lower line) effectors was assayed against the following targets: LG-15 (○), LG-46 (□), and LG-6 (▲) PHA-lymphoblasts as described for Fig. 2.
gp96 complexed in vitro with vesicular stomatitis virus antigenic peptides can interact with murine macrophages, leading to the cross-representation of the antigenic peptide by class I molecules of murine macrophages and peptide-specific activation of a MHC-restricted mouse CD8⁺ T cell line (22). We show in this study that both Xenopus hsp70 and gp96 can generate a CTL response specific against minor H-Ags. As in the mouse, neither Xenopus hsp70 that is free of peptide nor cognate gp96-peptide complexes elicit any CTL activity. In addition, CTL generated by gp96 or hsp70-peptide complexes kill only MHC-compatible targets derived from the same minor H-locus disparate genotype as the clone from which the HSPs were purified. This strongly suggests that the CTL response has been generated against chaperoned minor-H antigenic peptides and that these exogenous peptides have been channeled in the Ag presentation pathway of the host.

Strikingly, gp96 is able to generate responses against MHC class I-negative tumor in naturally class I-deficient larvae. However, in this case, the response does not appear particularly dependent on the presence of antigenic chaperoned peptide because immunization with gp96 purified from normal tissue is as potent as tumor-derived gp96 in evoking inhibition of tumor growth. Although in mice immunization with gp96 primarily stimulates CD8⁺ T cells, CD4 T cells (8) and NK cells (4) also appear to be recruited. These data and other in vitro studies (5, 6) suggest

**FIGURE 5.** Immunogenicity of tumor-derived gp96 in naturally MHC class I-deficient larval hosts. A–C, Effect of gp96 immunization on tumor growth after challenge. LG-15 larvae blocked at premetamorphic stage 55 with sodium perchlorate were primed i.p. with APBS (C, control), 1,000–100,000 irradiated 15/0 tumor cells, 15/0 total protein (T; 200 ng), 15/0 tumor-derived gp96, or normal LG-15 tissue-derived gp96 (24 or 120 ng). Doses as described in the figure were administered twice after a 2-wk interval. Two weeks after the last injection, larvae were challenged i.p. with 5 × 10⁴ 15/0 tumor cells. Two weeks later, the number of tumor cells per microliter of collected peritoneal fluid was determined for each animal. Group means (horizontal bars) and Student’s t test p values are given. D, Effect of gp96 immunization on survival after tumor challenge. Survival of LG-15 larvae used in panel A3 was monitored daily until all animals died. The percentage of group survival at each day postchallenge is shown (control, 12 individuals; experimental, 12 individuals). Differences in survival were statistically significant (Mantel-Cox nonparametric test; StatView) for control versus LG-15 liver-derived gp96 (p = 0.027) and control versus 15/0-derived gp96 (p = 0.001), but not between the two gp96 immunized groups (p > 0.46).
that HSP per se could induce nonpeptide-specific innate im-
mune responses mediated by enhanced cytokine production and
nonspecific killing. The recent characterization of a mouse B cell
line that is deficient in gp96 is of interest in this regard. This
gp96-deficient line is unresponsive to bacterial endotoxin (owing
to a lack of Toll receptor surface expression), but it is unaffected
by other stress conditions like temperature or glucose deprivation
(58). This suggests that, at least in higher organisms, gp96 may
have become specialized by responding to stressors associated
with pathogens rather than physical parameters.

Although the immunogenicity of tumor- and non-tumor-
derived gp96 in the present experiments with larvae does not appear
to be statistically different, a more potent response of HSP complexed with tumor-derived peptide cannot be com-
pletely excluded. In contrast to hsp70, peptides complexed to
gp96 can only be eluted by harsh treatments (e.g., heat or acid
treatment) that profoundly affect its conformation and its im-
umunological properties. Our recent cloning of
Expression 11:186.

References

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Heat shock proteins come of age: primitive functions acquire new roles in an
adapted world. Immunity 8:657.


surrounding the endoplasmic reticulum chaperone GRP94/gp96. Curr. Opin.
Immunol. 10:103.

y of tumors with autologous tumor-derived heat shock protein preparations.
Science 278:117.

Heat shock protein 72 on tumor cells a recognition structure for NK cells. J.

but not apoptotic cell death releases heat shock proteins, which deliver a partial
maturation signal to dendritic cells and activate the NF-κB pathway. Int. Immun.
12:1539.

transfer peptides during antigen processing and CTL priming. Immunogenetics
40:93.

major histocompatibility antigen-disparate skin grafts and acquisition of tolerance to thymus donor anti-
gens in the metamorphosing frog, Xenopus laevis. Transplantation 39:223.


M. Welsh. 1998. Immunization with a lymphocytic choriomeningitis virus pep-
tide mixed with heat shock protein 70 results in protective antiviral immunity.
J. Exp. Med. 186:1315.

11. Tochinai, S., and C. H. Katagiri. 1975. Complete abrogation of immune re-
sponses against xenogeneic antigens by immunization with the heat shock

12. Lammert, E., D. Arnold, M. Nijenhuis, F. Momburg, G. Hammerling, J. Brunner,
type stress protein gp96 binds peptides translocated by TAP. Eur.
J. Immunol. 27:923.

in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor im-

H. Schuld. 1999. Cutting edge: receptor-mediated endocytosis of heat shock pro-


