Cell Surface Expression of the Endoplasmic Reticular Heat Shock Protein gp96 Is Phylogenetically Conserved

Jacques Robert, Antoine Ménoret and Nicholas Cohen

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Jacques Robert, Antoine Ménoret, and Nicholas Cohen

In mammals, the heat shock protein gp96 complexed to antigenic peptides elicits T cell adaptive immunity. By itself, however, gp96 can evoke responses that are characteristic of innate immunity. Interestingly, this protein, which resides in the endoplasmic reticulum, is expressed on the surface of certain mouse tumor cells. Given that heat shock proteins are highly conserved, we investigated whether the cell surface expression of gp96 is also evolutionarily conserved. Our data reveal that gp96, most likely containing the endoplasmic reticulum retention motif (KDEL), is expressed on the surface of three different Xenopus lymphoid tumor cell lines, each derived from a different spontaneously arising thymic tumor. Levels of expression differ among the tumor lines tested, with more immunogenic tumors expressing greater amounts of surface gp96. Moreover, a high level of gp96 surface expression is detectable on a subset of Xenopus normal nontransformed splenic lymphocytes (mainly surface IgM+ B cells) but not on other normal cells, including macrophages and nucleated erythrocytes. Surface expression of a gp96 protein homologue occurs also on some, but not all, T and B cell clones derived from peripheral blood cells of the channel catfish, as well as on lymphocyte-like cells, but not on erythrocytes from the hagfish, a primitive agnathan vertebrate lacking markers of an adaptive immune system. gp96 is actively directed to and retained on the plasma membrane of Xenopus lymphocytes and tumor cells and hagfish lymphocyte-like cells by a process that requires vesicular transport. This selective surface expression of gp96 on some immune cells from different vertebrate classes is consistent with an ancestral immunological role of gp96 as danger-signaling molecule. The Journal of Immunology, 1999, 163: 4133–4139.
2% type I collagenase (C-2674, Sigma, St. Louis, MO), and dissociated cells were collected in physiological saline. Kidney mononuclear leukocytes (5–10 × 10^6 cells/ml) that microscopically resembled lymphocytes from higher vertebrates were separated from erythrocytes by centrifugation for 30 min at 1500 × g at 4°C on a 28% Percoll cushion (hagfish osmolarity). a) pT cell lines and surface IgM (slgM) B cell lines from channel catfish (Ictalurus punctatus) were kindly provided by Dr. Norman Miller (University of Mississippi Medical Center, Jackson, MS) (19). Freshly harvested Xenopus spleens were dissociated, and lymphocytes were purified with Histopaque 1.083 (Sigma); the three lymphoid tumor cell lines (B3B7, fl-2, and 15/0), which were derived from spontaneously arising thymic tumors in different strains or clones of adult Xenopus, were maintained in vitro (20, 21) before analysis. Isomolar PBS for each species was prepared from mammalian PBS.

### Monoclonal Abs

The following Xenopus-specific mouse mAbs were used: 10A9 directed against IgM (22); X71, specific for CTX (a cortical thymocyte-specific Xenopus cell surface receptor that is also present on the tumor cells used in this study (23)); AM22, specific for CD8-equivalent Ag (24); and TB17, an anti-actin mouse mAb (25). The anti-actin mouse mAb AC-40 was obtained from Sigma, the mouse mAb 10C3 specific for the KDEL C-termin al ER retention signal was purchased from StressGen (Biotechnologies, Victoria, British Columbia, Canada), and the rat mAb SPA-850 specific for gp96 (clone 9G10) was purchased from Neomarkers (Fremont, CA). This Ab recognizes a Xenopus glycoprotein of 98 kDa that can be purified using the same protocol for mouse gp96 (12, 14); four of the five carboxy-terminal amino acid residues of this 98-kDa glycoprotein are identical with those of mouse gp96 (our unpublished observations).

### Flow cytometry

Samples of 10^5 cells were incubated with hybridoma supernatants or purified Abs followed by fluorescein-labeled goat anti-mouse or rabbit anti-rat IgG F(ab')_2 (Southern Biotechnology Associates, Birmingham, AL), preadsorbed twice on Xenopus erythrocytes, and analyzed on an Elite flow cytometer (Beckman Coulter, Fullerton, CA) (23). Dead cells, detected by propidium iodide, were gated out. For two-color flow cytometry, spleen cells were first stained with rat anti-gp96 mAb followed by FITC-conjugated goat anti-rat (F(ab')_2) Ab (Southern Biotechnology Associates) that had been preadsorbed on mouse and Xenopus cells. Cells were then stained with Xenopus anti-IgM 10A9 mAb followed by PE-conjugated goat anti-mouse (F(ab')_2) Ab preadsorbed on rat and Xenopus cells. The two secondary Abs are not cross-reactive as certified by the supplier (Southern Biotechnology Associates); this was confirmed using isotype-matched rat and mouse control Abs.

### Cell surface labeling and immunoprecipitation

Procedures for cell surface biotinylation, lysis in Nonidet P-40, and immunoprecipitation with protein G have been detailed elsewhere (21). Before and after labeling, cells were extensively washed three times in PBS (at the appropriate amphibian or fish osmolarity) that contained 1% BSA. Cell death, determined before lysis by trypan blue dye exclusion, was never >5%. Biotinylated cell surface lysates (corresponding to ~5 × 10^6 cells) were precultivated for 1 h at 4°C with 30 μl/mg of protein G. A total of 100 μl of such precleared lysates (corresponding to ~5 × 10^6 cells) were incubated overnight at 4°C either with 100 μl of mAb supernatants and 30 μl of protein G or with a mixture of 3 μl of anti-gp96 mAb, 3 μl of rabbit anti-rat Ab (Sigma), and 30 μl of protein G. Immunoprecipitates were separated on 7.5% SDS-PAGE gels under reducing conditions and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). Biotinylated proteins were revealed using HRP-conjugated streptavidin and chemiluminescence reagents from Amersham (Arlington Heights, IL). Nonbiotinylated proteins were detected after reprobing the membrane with specific Ab followed by a secondary rabbit anti-rat HRP-conjugated Ab. Enrichment of IgM-splenic lymphocytes after surface biotinylation was performed using sheep anti-mouse magnetic beads adsorbed with Xenopus anti-IgM 10A9 mAb according to the manufacturer’s suggested protocol (Dynal, Fort Lee, NJ).

### Cell surface re-expression assay

The protocol described by Wiest et al. (10) was used. Briefly, cells were incubated with pronase (0.4 mg/ml final concentration, P-6911, Sigma) for 45 min at 26°C with occasional agitation; digestion was quenched for 10 min on ice with 2.5% BSA (final concentration) and 10 mg/ml of DNase (final concentration). Cells were washed once with 5% BSA in PBS and then incubated in 1% BSA in PBS with 0.1 mM PMSF and 0.05 mM TLCK Ne-p-tosyl-l-lysine chloromethyl ketone (both protein inhibitors) for 10 min on ice. After an additional wash, cells were put back in culture for 4 h at 26°C in either medium alone (for hagfish, Iscove-derived mammalian medium supplemented with 1.2% NaCl, 5% FBS, and 5% hagfish serum) or medium with 1 μg/ml of brefeldin A (BFA).

### Results

#### Surface expression of gp96 on tumor cells

Our previous characterization of the 15/0, fl-2, and B3B7 Xenopus tumors (20, 26, 27) and the present studies suggest a correlation between gp96 surface expression and immunogenicity (see Table I). The level of gp96 surface expression of the different Xenopus lymphoid tumor cell lines derived from independent spontaneously arising thymic tumors was analyzed by flow cytometry. Each of the three lymphoid tumor cell lines showed a reproducible but different level of gp96 surface expression (Fig. 1A). The least immunogenic tumor, 15/0, expresses the lowest level of surface gp96, whereas the highly immunogenic B3B7 tumor line expresses the most. The fl-2 cell line, which has an intermediate level of gp96 surface expression, is tumorigenic in larval (or less immunogenic) hosts but not in fully grown adult MHC identical hosts. Furthermore, tumor rejection of fl-2 cells is mediated by a thymus-dependent immune response against tumor-specific Ags that develop during metamorphosis (29). Another tumor clone (15/40, clone A1) that recently lost its tumorigenicity displays levels of surface

### Table 1. Characteristics of three different Xenopus thymic tumor cell lines

<table>
<thead>
<tr>
<th>Name of the Tumor Clone</th>
<th>B3B7</th>
<th>fl-2</th>
<th>15/0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic background</td>
<td>MHC homozygous</td>
<td>fl strain</td>
<td>LG-15 (a/c)</td>
</tr>
<tr>
<td></td>
<td>partially inbred</td>
<td></td>
<td>isogenic clones</td>
</tr>
<tr>
<td>T cell surface markers</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>(CD8, CD5, pan T cell)</td>
<td></td>
<td></td>
<td>21, 28</td>
</tr>
<tr>
<td>Ig mRNA</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ig protein</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Classical MHC class 1a</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>mRNA and protein</td>
<td></td>
<td></td>
<td>20, 26</td>
</tr>
<tr>
<td>Nonclassical MHC class 1b</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>mRNAs</td>
<td></td>
<td></td>
<td>20, 26, 27</td>
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<tr>
<td>MHC class II protein</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Surface gp96</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Tumorigenicity in the</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>syngenic larvae host</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tumorigenicity in the</td>
<td></td>
<td></td>
<td>20, 26, 27</td>
</tr>
<tr>
<td>syngenic adult host</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>

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sorbed twice on Xenopus cells stained by propidium iodine were gated out.

and anti-CD8 mAb. The negative control for gp96 was obtained with a mAb specific for gp96, but not in control samples immunoprecipitated with a mAb specific for gp96, but not in control samples immunoprecipitated with purified rat IgG or rat serum (Fig. 2A). The possibility that the biotinylation may have artifically labeled intracellular proteins released from a minor fraction of dead cells that are ubiquitous in cultures is unlikely, because an anti-actin protein by pronase (Fig. 3) revealed that as in the case of tumor lysates with anti-KDEL mAb revealed weak but reproducible surface expression of intact gp96 (Fig. 2B) and two other KDEL-expressing proteins of 78 kDa and 160–160 kDa that might be the two hsp70, BiP (grp78) and grp170, respectively (30). Interestingly, immunoblotting of 15/0 total cell lysates with the anti-KDEL Ab revealed at least four other proteins of 160–160, 60, 50, and 40 kDa. It appears that in addition to gp96, some, but not all, ER-resident proteins end up at the cell surface. Results similar to those obtained with 15/0 cells were also recorded for the B3B7 and ff-2 tumor cell clones (data not shown). The extensive washes before and after biotinylation were designed to eliminate any adventitious deposition of gp96 released in the medium by dead cells during the in vitro culture. To completely eliminate this possibility, 15/0 cells were incubated with an excess of biotinylated cell lysate (obtained by successive freeze/thaw cycles to avoid the use of detergent). No signal was detectable for cells incubated with biotinylated lysates after immunoprecipitation, whereas large amounts of biotinylated gp96 were detectable from the lysate (Fig. 2C).

Because passive loading from free extracellular gp96 can be ruled out as a source of surface gp96, we subsequently examined whether gp96 surface expression requires cell integrity by monitoring gp96 reappearance on the cell surface after complete digestion of all surface proteins with pronase (Fig. 3). After such a treatment (that also eliminates dead cells), surface gp96 was undetectable (Fig. 3); after 4 h, however, it could again be detected. Similar re-expression at 4 h after extracellular digestion was monitored by flow cytometry (data not shown) of cells stained for CTX, a well-characterized Xenopus transmembrane protein (23).

In addition, surface re-expression of gp96 was abrogated in the presence of BFA (Fig. 3), which indicates that the presence of gp96 on the cell surface results from an active process that is dependent upon the translocation of protein from the ER to the Golgi. Note that treatment with pronase and BFA does not affect the overall abundance of gp96, because similar amounts of gp96 could be detected in lysates by Western blotting with anti-gp96. Similar BFA-sensitive re-expression after pronase treatment was obtained with the B3B7 tumor line (data not shown).

A final argument that the precipitation of surface gp96 from 15/0 cells is not an artifact of biotinylation or cross-reaction but rather is a selective phenomenon restricted to some type and/or differentiation step of cells is that no such signal could be obtained from the surface labeling of normal Xenopus erythrocytes, peritoneal macrophages (Figs. 1 and 4), and most splenic lymphocytes (Fig. 1). Indeed, Xenopus erythrocytes that are nucleated and express surface MHC class I Ags also do not express surface gp96 (Figs. 1 and 4). In immunoprecipitation experiments, reprobing membranes with the anti-gp96 Ab revealed a significant amount of precipitated intracellular unbiotinylated gp96 (Fig. 4, bottom panel).

Surface expression of gp96 on a subpopulation of immune cells

Interestingly, surface gp96 was detected by immunoprecipitation of surface-biotinylated 15/0 tumor cells. A major protein of 98–99 kDa was specifically detected in the sample immunoprecipitated with a mAb specific for gp96, but not in control samples immunoprecipitated with purified rat IgG or rat serum (Fig. 2A). The possibility that the biotinylation may have artifically labeled intracellular proteins released from a minor fraction of dead cells that are ubiquitous in cultures is unlikely, because an anti-actin mAb did not precipitate any biotinylated material (data not shown). Because gp96 is normally a resident protein of the ER and contains the retention signal KDEL, we wondered whether surface expression of gp96 could have resulted from the absence of this signal caused by C-terminal degradation of a fraction of the gp96 protein. Immunoprecipitation of surface-biotinylated 15/0 tumor cells stained by propidium iodine were gated out.

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minor (5–9%) subset was strongly positive (about a two-log difference from the rat IgG control; Fig. 1). When viewed microscopically, these viable gp96\(^{+}\) small lymphocytes exhibited typical punctuate surface staining. Further analysis by two-color flow cytometry revealed that the majority (>90%) of these gp96\(^{+}\) small lymphocytes were also sIgM\(^{+}\) (Fig. 5A). No significant staining of this gp96\(^{+}\) IgM\(^{+}\) cell subset was obtained with either Xenopus anti-CD8 mAb or anti-CD5 mAb, both of which exclusively detect Xenopus T cells. To test whether this positive surface staining was the result of artifactual deposition of cytoplasmic gp96 and/or IgM released by dead cells, spleen cell suspensions were kept on ice for 2 h before the washing and staining steps. Although this treatment increased the numbers of dead cells, it did not increase the fraction of surface gp96\(^{+}\) splenocytes. Instead, it resulted in a decrease of ;25% of surface gp96\(^{+}\) cells (data not shown). Because spleens also contain a large number of macrophages, we purified them by adherence on a glass microscope coverslide and stained them with either anti-gp96, anti-class I, or class II mAbs followed by an FITC-conjugated secondary Ab before fixation with 2% paraformaldehyde. No fluorescence signal was detected with anti-gp96, whereas a bright surface staining pattern was obtained with both anti-MHC mAbs (data not shown). To further substantiate the two-color flow cytometric data, IgM\(^{+}\) spleen lymphocytes were positively selected with anti-IgM-coated magnetic beads after surface biotinylation. The amount of surface gp96 precipitated from this enriched IgM\(^{+}\) subset was markedly increased relative to the total or partially depleted splenocyte populations (Fig. 5B), whereas similar signals in all samples were detected by immunoprecipitation with Xenopus-specific anti-class I mAb.

Surface expression of gp96 on immune cells from other classes of vertebrate

If surface expression of gp96 on normal Xenopus lymphoid cells is biologically relevant, then we hypothesized that this hsp should be
present on the surface of normal cells from representatives of phylogenetically more primitive vertebrates. To explore this possibility, we took advantage of the high degree of conservation of the epitope recognized by the anti-gp96 mAb (i.e., it reacts with a single monomorphic protein of 96 kDa on Western blots in all vertebrate species tested, including agnathans (31)).4 Also, the same 96-kDa monomer can be detected by Western blotting with an anti-KDEL mAb. Immunoprecipitation of surface-biotinylated gp96, MHC class I, and IgM was performed on total spleen cells, spleen cells depleted of sIgM-expressing cells, and spleen cells enriched for sIgM-expressing B cells as described in Fig. 3. Each lane represents the material precipitated from 7 × 10^6 cells.


FIGURE 5. Expression of gp96 at the surface of IgM^+ B spleen cells in Xenopus. A, Two-color flow cytometry of spleen cells stained with rat anti-gp96 mAb followed by FITC-conjugated goat anti-rat (F(ab')_2) Ab (preadsorbed on mice and Xenopus cells) and with Xenopus anti-IgM 10A9 mAb followed by PE-conjugated goat anti-mouse (F(ab')_2) Ab (preadsorbed on rat and Xenopus cells). Note that the minor fraction (usually <1%) that was single positive for gp96 was also detected using rat IgG2a isotype as a negative control; therefore, this fraction is likely to be non-specific or autofluorescent. B, Immunoprecipitation of surface-biotinylated gp96, MHC class I, and IgM was performed on total spleen cells, spleen cells depleted of sIgM-expressing cells, and spleen cells enriched for sIgM-expressing B cells as described in Fig. 3. Each lane represents the material precipitated from 7 × 10^6 cells.

FIGURE 6. Expression of gp96 on the surface of some, but not all, catfish T and B cell clones. Surface-biotinylated lysates were immunoprecipitated from two different sIgM^+ B cell clones (1G8, 3B11) and two oβT cell clones (28S1, 43TA) from the channel catfish, I. punctatus. Results are representative of two different experiments (Fig. 7) but not on erythrocytes that are nucleated as they are in amphibians. Moreover, as in Xenopus, the gp96 hagfish homologue is loaded at the cell surface by an active mechanism that is sensitive to BFA (Fig. 7).

Discussion

The hsp gp96 has been described as a classical ER-resident protein whose expression on the surface of some mouse tumor cells has been considered atypical (3). Here, we show surface expression of gp96 on tumor cells of the frog, Xenopus. As in mice, the gp96 localized on the surface of Xenopus tumor cells is derived from the pool of endogenous gp96 synthesized by the cells, rather than from an adventitious deposition. The mechanism by which gp96 is directed and retained on the plasma membrane is still unknown. Detection of surface gp96 with a mAb specific for the C-terminal ER retention signal (KDEL) suggests that escape of C-terminal degraded gp96 is unlikely. The two other proteins of 78 kDa and 160–180 kDa are likely to be BiP (grp78) and grp170 (a newly identified ER protein), respectively. grp170 belongs to the hsp family, shows homology with both hsp70 and hsp110, binds ATP, and possesses a carboxyl terminal retention sequence (30).

The process by which gp96 is expressed at the cell surface was further characterized using freshly harvested Xenopus splenic lymphocytes as well as MHC class I-negative tumor lymphoid cell lines (B3B7, 15/0). Although digestion of surface proteins by pro tease initially resulted in the loss of cell surface gp96, re-expression of this hsp at 26°C was detectable as early as 4 h after pronase treatment for both tumor cells and nontransformed splenocytes. Furthermore, this reappearance was abrogated by inhibiting protein translocation from the ER to the Golgi with BFA. These experiments rule out any contribution to surface expression by dead cells and suggest that surface expression results from an active process involving vesicular transport. It is noteworthy that this inhibitor interferes with re-expression of surface proteins (10) but not with internal labeling of cytosolic proteins in dying cells (33).

Our previous characterization of the 15/0, ff-2, and B3B7 Xenopus tumors and our present studies suggest a positive correlation between gp96 surface expression and immunogenicity (see Table I). No correlation between the immunogenicity of these three tumors and the level of surface MHC class I and class II molecules or other molecules was found. However, 15/0, the least immunogenic tumor, expresses the lowest level of surface gp96, whereas the highly immunogenic B3B7 tumor line expresses the most. The ff-2 cell line, which has an intermediate level of gp96 surface expression, is tumorigenic in larval but not in fully grown adult histocompatible hosts. Furthermore, tumor rejection of ff-2 cells is mediated by a thymus-dependent immune response against tumor-specific Ags that develop during metamorphosis (29). Another tumor clone (15/40.A1) that has recently lost its tumorigenicity expresses levels of surface gp96 that are comparable with the highly immunogenic B3B7 tumor (our unpublished observations).
addition to this antigenic peptide-specific process, hsps such as gp96 have been shown to play a role in Ag presentation and in the generation of classical adaptive immunity (31). gp96 has been proposed to play a role in innate immunity as molecular messengers of cell death (11, 37) or "danger" (17).

The activation of defense responses by hsp per se and/or hsp complexed to peptides is thought to be triggered by the release of hsp by dead or apoptotic cells. However, it is possible that modulated surface expression of such molecules may also play a role in this activation. It is with this in mind that we propose, and are currently testing, the hypothesis that gp96 surface expression represents a vestige of an ancestral system of Ag presentation and/or immune surveillance.

In summary, our results strongly suggest that cell surface expression of the ER-resident molecular chaperone gp96 is not unique to some mammalian tumors but rather, is reflective of a more general biological phenomenon that occurs on populations of normal lymphoid cell populations from phylogenetically diverse vertebrates, including the Agnatha, which lack an adaptive immune system.

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References


