Bacterial stimulation upregulates the surface expression of the stress protein gp96 on B cells in the frog *Xenopus*

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Abstract The presence of the soluble intracellular heat shock protein gp96 (an endoplasmic reticulum resident protein) at the surface of certain cell types is an intriguing phenomenon whose physiological significance has been unclear. We have shown that the active surface expression of gp96 by some immune cells is found throughout the vertebrate phylum including the Agnatha, the only vertebrate taxon whose members (lamprey, hagfish) lack an adaptive immune system. To determine whether gp96 surface expression can be modulated by pathogens, we investigated the effects of in vitro stimulation by purified lipopolysaccharide (LPS) and the heat-killed gram-negative bacteria, *Escherichia coli* and *Aeromonas hydrophilia*. Purified *Xenopus* B cells are readily activated and markedly proliferate in vitro in response to the heat-killed bacteria but not to purified LPS. Furthermore, messenger ribonucleic acid, and intracellular and surface protein expressions of both gp96 and immunoglobulin were upregulated only after activation of B cells by heat-killed bacteria. These data are consistent with an ancestral immunological role of gp96 as an antigen-presenting or danger-signaling molecule, or both, interacting directly with antigen-presenting cells, T cells, or natural killer cells, (or all), to trigger or amplify immune responses.

INTRODUCTION

Upregulation of heat shock or stress proteins (Hsp) is a response shared by all types of cells, from those of prokaryotes to those of eukaryotes, to face various environmental stressors including infection. The importance of this type of response is illustrated by the high degree of phylogenetic conservation of members within each Hsp family. For example, the overall amino acid similarity among Hsp90 members is greater than 50% (Gupta 1995; Nicchitta 1998).

In mammals and in the frog *Xenopus*, Hsps are involved in activation of the adaptive as well as the innate immune systems (review in Srivastava 2002; Robert 2003). Specifically, antigenic peptides chaperoned by Hsps stimulate potent T-cell responses after they undergo receptor-mediated endocytosis by antigen-presenting cells (APCs) and representation by APC's major histocompatibility

complex (MHC) class I (Suto and Srivastava 1995; Binder et al 2000a). Hsps also induce an innate type of response (eg, secretion of tumor necrosis factor- α and interleukin-1 β [Basu et al 2000]) in an antigenic peptide-independent manner. Several studies suggest that because of their abundance, Hsps are the major protein species released into the extracellular milieu by cell necrosis but not by apoptosis (Basu et al 2000; Binder et al 2000b; Lehner et al 2000). Thus, Hsps associated with nonprogrammed cell death resulting from viral infection and cancer may be considered a "danger" signal (Matzinger 1994).

Gp96, a member of the Hsp90 family, is the major resident protein of the endoplasmic reticulum (ER) (review in Csermely et al 1998). Like other ER-resident proteins, gp96 contains a KDEL (Lys-Asp-Glu-Leu) motif at its carboxyl terminus, which is a retention-retrieval signal from the golgi to the ER (Koch et al 1986; Peter et al 1992). We have shown in the frog *Xenopus* that even though gp96 contains the ER-retention motif, it is actively expressed at the surface of a subset of immunoglobulin IgM⁺ B cells and several lymphoid tumor cell lines by an active pro-

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cess involving vesicular trafficking (Robert et al 1999). This phenomenon is relatively cell type specific because surface gp96 is undetectable on nontransformed *Xenopus* erythrocytes, splenic and peritoneal macrophages, and fibroblasts. We have found a similar gp96 surface expression on some, but not all, catfish lymphoid lines and on lymphocytelike cells of the Pacific hagfish (Robert et al 1999). In mice, surface expression of gp96 has been observed in various tumor cells (Altmeyer et al 1996), lipopolysaccharide (LPS)-stimulated B cells (Banerjee et al 2002), and a subset of murine immature thymocytes (Wiest et al 1997) but not in normal embryonic fibroblasts (Altmeyer et al 1996). Some in vitro evidence suggests that gp96 expressed at the surface of LPS-stimulated murine B cells may activate Th2 cells (Banerjee et al 2002). Furthermore, the physical interaction of gp96 with a Toll-like receptor (TLR) of a B cell line was described in mice (Randow and Seed 2001), suggesting the possibility of surface coexpression of gp96-TLR complex. Altogether, these observations are consistent with the idea that gp96 surface expression may play a role in immune surveillance.

The objective of this study was to investigate the modality of surface expression of gp96 by *Xenopus* B cells that are stimulated with LPS and heat-killed gram-negative bacteria (*Escherichia coli*, *Aeromonas hydrophila*). Our results reveal that intracellular and surface expression of gp96 is upregulated by such stimulation.

MATERIALS AND METHODS

Animals

Adult (2–3 year old) outbred *Xenopus* were either obtained from our breeding colony or purchased from Xenopus I (Dexter, MI, USA).

B-cell purification and stimulation

B cells from each outbred animal were positively selected with the *Xenopus*-specific anti-IgM 10A9 monoclonal antibody (mAb) (Hsu and Du Pasquier 1984) and magnetic microbeads (MACS; Miltenyi Biotec, Auburn, CA, USA) coupled with mouse-specific anti-IgG following the manufacturer's instructions. Cells from each animal were grown separately in 24-well plates (1 to 2×10^6 cells/2 mL/well) and were incubated for 1–6 days with LPS (1 – 100 μ g/mL; *E. coli* serotype 0111:B4, Sigma L4130) or with heat-killed bacteria. *E. coli* (XL1-blue, Stratagen, La Jolla, CA, USA) cultured overnight at 37°C and *A. hydrophila* (ATCC 7965) cultured for 7 days at room temperature were boiled for 30 minutes, spun, and resuspended in 0.1 volume of *Xenopus* cell culture medium (Robert and Du Pasquier 1996). B cells were stimulated with the

equivalent of 1×10^8 to 1×10^6 bacteria/mL of culture (1 – 100 μ g protein/mL).

Flow cytometry

Total or selected lymphocytes (10^5 cells) were stained with 1 μ g/mL of purified *Xenopus*-specific biotinylated mAb followed by phycoerythrin (PE)-conjugated or fluorescein isothiocyanate (FITC)-conjugated streptavidin (Pharmagen, San Diego, CA, USA). The following mAb reagents were used: biotinylated anti-CD8 (AM22; Flajnik et al 1991) and IgM (6.16, Bleicher and Cohen 1981); the stained cells were then analyzed by flow cytometry on a FACSCalibur; 10 000 events were collected.

Cell surface biotinylation

Cells were biotinylated as already published (Robert and Du Pasquier 1996; Robert et al 1999) with sulfosuccinimidyl-6-(biotiamido) Hexanoate (NHS-LC-biotin) (0.5 mg/mL)–amphibian phosphate buffered saline (APBS) solution and incubated for 30 minutes. The biotinylation reaction was stopped by washing 3 times with Lys-APBS. Cells were then lysed for 20 minutes with NP-40 lysis detergent and treated with rat anti-gp96 mAb (SPA-850, StressGen Biotechnologies, Victoria, British Columbia, Canada) and rabbit anti-rat IgG secondary antibody and immunoprecipitated with protein G. For control purposes, IgM was precipitated with the *Xenopus*-specific anti-IgM 10A9 mAb (Hsu and Du Pasquier 1984). Total amount of protein was determined by a colorimetric method (Bicinchoninic acid [BCA]; Pierce, Rockford, IL, USA).

Reverse transcriptase–polymerase chain reaction

Cytoplasmic ribonucleic acid (RNA) and first-strand complementary deoxyribonucleic acids (cDNAs) were prepared as previously published (Robert et al 2001b). For each polymerase chain reaction (PCR) (30 μ L total volume), 3 μ L of 1.25 mM diethylnitrophenyl thiophosphates, 3 μ L of $10\times$ PCR buffer, 1 μ L of each primer, 2 U of *Taq* DNA polymerase (Life Technologies, Grand Island, NY, USA), and 1 μ L of cDNAs were used. Tubes were then set for 35 cycles of denaturation for 45 seconds at 95°C , annealing for 45 seconds at 56°C , and extension for 10 minutes at 72°C . *Xenopus*-specific primers were: gp96 5'-GTTGTAAGCAGGGAAGAGG-3' and 5'-TGTCTCAGTCTTGCTGCTCCACAC-3' (277 bp); C μ 5'-GCAATGCCAAACACCTGG-3' and TCTT 5'-CTCTTGTAAGGAACCCG-3' (350 bp); elongation factor- α 5'-CCTGAATCACCCAGGCCAGATTGGTG-3' and 5'-GAGGGTAGTCTGA GAAGCTCTCCACG-3' (223 bp).

Fig 1. Positive selection of immunoglobulin IgM⁺ B cells. Splenic B cells were positively selected with the *Xenopus*-specific anti-IgM 10A9 monoclonal antibody (mAb) and magnetic microbeads coupled with mouse-specific anti-IgG. Positively selected (B⁺) and the remaining depleted cells (B⁻) (10⁵ cells) were then stained with 1 µg/mL of another *Xenopus*-specific biotinylated IgM mAb (6.16) or with anti-CD8 (AM22) mAb as positive control followed by phycoerythrin-conjugated or fluorescein isothiocyanate-conjugated streptavidin. The stained cells were then analyzed by flow cytometry on a FAC-SCalibur; 10 000 events were collected. Isotype control staining is shown in the upper panel (stripped lane).

RESULTS

Stimulation of B cells by purified LPS, *E coli*, and *A hydrophila*

Compared with mammals, *Xenopus* B cells are only poorly reactive in vitro to purified LPS (Bleicher et al 1983). Therefore, we determined whether a whole-bacteria extract might provide better stimulation (eg, blast formation, proliferation). Splenic IgM⁺ B cells were purified from naive outbreds by magnetic sorting using the *Xenopus*-specific anti-IgM mAb 10A9 and anti-mouse Ig-conjugated microbeads. As determined by flow cytometry using another anti-IgM mAb (6.16; Fig 1), selected B cells were typically >95% pure. Purified naive B cells stimulated for 1 day in culture with heat-killed *E coli* or *A hydrophila* began to blast (ie, appeared larger and had a more rounded morphology) and form large cell aggregates. In contrast, cells cultured in medium alone remained small and did not aggregate. Effects on proliferation were monitored for several days by counting cells and determining cell death by trypan blue exclusion using various concentrations of heat-killed *E coli* (1 × 10⁷ to 1 × 10⁹ bacteria/mL corresponding to 1–100 µg total protein/mL of culture). The best response was obtained with 1 × 10⁸ to 1 × 10⁹ bacteria/mL (10–100-µg/mL). Consistent B cell proliferation in the presence of heat-killed bac-

Fig 2. Cell proliferation. (A) Cell counts from 4 separate experiments at different days of culture under different condition are expressed as fold increase from the starting cell number at day 0. (B) Thymidine incorporation assays: 50 000 purified IgM⁺ B cells or leukocytes depleted of B cells were incubated, in triplicate, in 96-well plates for 2, 4, or 6 days with either medium alone (control) or medium containing LPS (50 µg/mL), heat-killed *Aeromonas hydrophila* (1 × 10⁹/mL), or heat-killed *Escherichia coli* (1 × 10⁹/mL). Cells were pulsed with [³H]thymidine ([³H]-TdR) for the last 20 hours at 26°C and harvested with a 96-well harvester (Betaplate, Wallac, Turku, Finland). Thymidine uptake was determined by scintillation spectrometry with a Matrix 96 direct beta counter (Packard, Meriden, CT, USA). Variation within triplicate groups was less than 10% of group mean counts per minute.

teria was noticeable after 2 days of culture and peaked at day 4–5 (Fig 2A). Cell death, minimal during the first few days of culture (less than 10%), increased after 6 days of culture (20–40%), in part because of overcrowding in culture wells. Although cell cultures could be maintained for up to 2 weeks, long-term cultures were not studied. In contrast to their response to heat-killed bacteria, the response of B cells to LPS occurred only at high (relative to mice) concentrations (50–100 µg/mL) of LPS. B cell-depleted cultures were not markedly affected by killed bacteria or LPS (ie, minimal proliferation and cell death were observed).

To substantiate these observations, in vitro proliferation, assayed by tritiated thymidine incorporation, was then performed on positively selected B cells with various amounts of antigens. B cells proliferated promptly (ie, significant DNA synthesis after only 2 days in culture) and steadily in response to killed *A hydrophila* and *E coli*. Thymidine incorporation assays also confirmed that *Xenopus* B cells were poorly responsive to LPS, as has already been published (Bleicher et al 1983).

To test whether stimulation by killed bacteria was an indirect effect mediated by macrophages or other APCs, adherent cells were removed by incubation for 1 hour on plastic dishes before purifying B cells. Removal of adherent cells did not significantly affect the B cell response to heat-killed bacteria. In contrast, spleen cells depleted of B cells showed only minimal thymidine incorporation (about 10 times less, Fig 2B), providing further indication that killed bacteria were specifically and directly stimulating B cells. Even at lower doses, bacteria were better stimulators than the highest dose of LPS used (Fig 2B). Also, heat-killed *E coli* added to cultures of B cells at the same concentration as *A hydrophila* elicited a comparable response (Fig 2B).

Effects of in vitro stimulation on gp96 expression

Given a reliable system to reproducibly and markedly stimulate B cells in vitro, we assessed gp96 surface expression on activated B cells by cell surface labeling and immunoprecipitation. Purified B cells, cultured in the presence of heat-killed *E coli* for 5–6 days, were surface biotinylated, and gp96 was immunoprecipitated with an anti-gp96 rat mAb. A major protein of ~100 kDa (the expected size of the gp96 monomer) was observed in samples incubated with anti-gp96 mAb but not in controls incubated with normal rat IgG (Fig 3A, upper panel). In addition, the level of expression of gp96 in *E coli*-treated B cells was approximately 10 times that of sham-treated controls, whereas cell death before biotinylation was comparable (>8%). In an extensive published study (Robert et al 1999), we ruled out that the gp96 cell surface signal results from biotinylation of damaged cells or from free gp96 in the medium that is passively loaded to the cells. Similar upregulation of surface gp96 on B cells was also obtained using *A hydrophila* (data not shown) and with lower amount of bacteria (1×10^8 bacteria/mL, 10 µg/mL protein, Fig 2B). In Figure 3, the band migrating around 85 kDa appears to be material that cross-reacts with the rabbit anti-rat secondary antibody (ie, it is also found in samples where anti-gp96 was replaced by normal rat IgG, Fig 2A, lane C) and degradation products of gp96 (detected by reprobing the membrane with anti-gp96 mAb, Fig 3B, left panel).

To determine the total amount of gp96 (surface biotinylated and nonbiotinylated intracellular gp96), the membranes were washed and reprobed with anti-gp96 followed by a horseradish peroxidase (HRP)-conjugated rabbit anti-rat secondary antibody (Robert et al 1999). On stimulation of B cells, the total amount of gp96 also increased, in similar fashion to surface gp96 (Fig 3A, bottom panel and Fig 3B, left panel). Another indication of the high state of activation of B cells exposed to bacteria is the marked increase of surface IgM (Fig 3D). Surface

upregulation of gp96 was already apparent after 1 day of stimulation (Fig 3C) but became high only after 5–6 days of stimulation. B cell stimulation with LPS also induced increased gp96 surface expression, but to a lesser extent, and it occurred later than stimulation with bacteria (ie, not detected after 3 days of stimulation, Fig 3C). Whereas some gp96 surface expression could be detected in B cell-depleted cultures, the signal was usually weak and did not increase on stimulation with bacteria (data not shown). Despite the increased amount of surface gp96 detected over several days of stimulation with heat-killed bacteria, we were unable to detect free gp96 in the culture medium by Western blotting even after protein was concentrated by acid precipitation (data not shown). Increased gp96 surface expression on bacterial stimulation has been observed under various conditions (ie, doses, duration of stimulation, bacterial species) involving more than 50 frogs without marked variation among individuals.

To further determine whether B cells exposed to heat-killed bacteria were also upregulating gp96 gene expression, the level of gp96 messenger RNA (mRNA) was determined by reverse transcriptase–polymerase chain reaction. Indeed, a higher amount of gp96 transcript was detected in B cells cultured for 5 days in the presence of heat-killed *E coli* or 50 µg/mL of LPS compared with cells cultured in medium alone. Moreover, on stimulation of B cells with *E coli* (Fig 4) or *A hydrophila*, (data not shown) IgM mRNA was detected in high amount, whereas no (or scant amount) transcript was detected in unstimulated or LPS-treated B cells. The absence of signal in unstimulated and LPS-treated culture was not due to cell death or RNA degradation because no significant cell death by trypan blue exclusion was observed and the message for the housekeeping gene Ef-1α as well as for gp96 was detected. However, the lower signal intensity of Ef-1α and gp96 suggests that B cells stimulated with large numbers of heat-killed bacteria (Fig 4, lane H) were less active after 6 days of culture.

DISCUSSION

It is not clear why in *Xenopus*, LPS is not as effective as heat-killed *E coli* or *A hydrophila* in activating B cells. It is possible that the modest responses obtained only at high concentrations of LPS (≥ 50 µg/mL) are in fact due to a contaminant (eg, lipoprotein) as already reported (Bleicher et al 1983). This would imply that *Xenopus* B cells are somehow defective in their LPS receptor. Poor responsiveness of *Xenopus* is further exemplified by the observation that intraperitoneal injection of 0.5 mg of LPS, which would be lethal for a human or a mouse, is well tolerated by *Xenopus* tadpoles and adults (Robert 2003). In any case, reliable and potent stimulation was

B-cell exposure to heat-killed *E.coli*

Fig 3. Gp96 surface expression. (A) Cell lysates from B cells cultured for 6 days in the presence of 10^9 /mL heat-killed *Escherichia coli* or in medium alone and surface biotinylated were precipitated with anti-gp96 rat monoclonal antibody (mAb) or with normal rat immunoglobulin G (IgG) as a negative control. Precipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. Biotinylated gp96 was detected by HRP-conjugated streptavidin and chemoluminescence. The membrane was reprobed with anti-gp96 and HRP-conjugated anti-rat secondary antibody to detect total (nonbiotinylated) gp96. (B) Similar experiment where B cells were stimulated for 5 days with either 10^9 /mL (H) or 10^6 /mL (L) heat-killed *E. coli*. (C) B cells were stimulated for 1, 4, or 6 days with 10^9 *E. coli* or 100 μ g/mL of lipopolysaccharide or cultured in medium alone for the period of time (control). (D) Cell lysates from B cells cultured for 6 days with 10^9 /mL heat-killed *E. coli* or in medium alone, surface biotinylated, and precipitated with anti-IgM mAb 10A9. In all experiments, cell death, determined by trypan blue exclusion, was less than 10%. The amounts of sample loaded on gel were adjusted to represent equivalent amounts of total protein (determined by BCA; Pierce) of the lysates.

obtained with heat-killed bacteria, providing a convenient way to investigate the expression modality of gp96.

LPS stimulation of mouse B cells induces gp96 surface expression, and as a result, activates Th2 cells (Banerjee et al 2002). Our data indicate that in *Xenopus*, stimulating B cells with heat-killed bacteria also markedly increases (10–50-fold) the amount of gp96 present at their cell surface over several days. Surface gp96 upregulation already occurs after an overnight stimulation, suggesting a rapid modulation without an absolute requirement for cell proliferation. Although we do not know whether all B cells

or only a subset are upregulating surface gp96, these data suggest that an overall increased fraction of the intracellular fraction of gp96 is accessing the extracellular compartment in response to bacterial stimulation. Although our results indicate that B cells stimulated for 6 days by heat-killed bacteria and LPS also upregulate gp96 gene expression, a more detailed study is needed to determine the association of this phenomenon, if any, with gp96 surface upregulation.

It has been proposed that by virtue of their abundance, Hsps are the major protein species released in the extra-

Fig 4. Gp96 reverse transcriptase–polymerase chain reaction. Cytoplasmic ribonucleic acid from 200 000 B cells stimulated for 6 days with 10% mL (H), 10% mL (M), 10% mL (L) *Escherichia coli* or with 100 mg/mL of lipopolysaccharide, or cultured in medium alone, were reverse transcribed. Aliquots of reverse-transcribed reaction were amplified (30 cycles) with primer pairs specific for *Xenopus* gp96, C μ , or for Ef-1 α as a positive control.

cellular compartment when cells die by necrosis but not by apoptosis (Melcher et al 1998; Basu et al 2000; Berwin et al 2001). According to this view, Hsps could be a “danger signal” (Matzinger 1994) associated with nonprogrammed cell death that occurs in viral infection or cancer. In *Xenopus*, increased gp96 was found at the surface of stimulated B cells but not in the culture medium (ie, no active secretion or necrosis). Given the dual ability of gp96 to chaperone antigenic peptides and to stimulate innate immune responses in *Xenopus* (Robert et al 2001a, 2002) as well as in mice (Basu et al 2000; Binder et al 2000b; Lehner et al 2000), elevated expression of surface gp96 induced by bacteria may play a role in immune surveillance by allowing B cells to trigger or enhance immune responses. Immunomodulation by surface gp96 has been obtained with transformed cells that express recombinant gp96 targeted to the cell surface by the addition of a transmembrane domain (Zheng et al 2001); these gp96 surface-positive tumor cells induced efficient dendritic cell maturation after cell-to-cell contact. Therefore, increased gp96 surface expression by activated immune cells may constitute a way other than necrosis to expose APC or T cells to Hsps. In this regard, it is tempting to speculate that surface gp96 may directly interact with receptors on the surface of other cells like T cells or APCs. In mammals, the uptake of gp96-peptide complexes is mediated by CD91, the receptor for α 2-macroglobulin (Basu et al 2001; Binder et al 2000a). Once we complete our characterization of the putative CD91 homologue in

Xenopus (Robert et al, in preparation), we will be in an excellent position to fully test this hypothesis.

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