The Endoplasmic Reticulum-resident Heat Shock Protein Gp96 Activates Dendritic Cells via the Toll-like Receptor 2/4 Pathway

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The heat shock protein Gp96 has been shown to induce specific immune responses. On one hand, this phenomenon is based on the specific interaction with CD91 that mediates endocytosis and results in major histocompatibility complex class I-restricted representation of the Gp96-associated peptides. On the other hand, Gp96 induces activation of professional antigen-presenting cells, resulting in the production of pro-inflammatory cytokines and up-regulation of costimulatory molecules by unknown mechanisms. In this study, we have analyzed the consequences of Gp96 interaction with cells expressing different Toll-like receptors (TLRs) and with bone marrow-derived dendritic cells from mice lacking functional TLR2 and/or TLR4 molecules. We find that the Gp96-TLR2/4 interaction results in activation of nuclear factor-kB-driven reporter genes and mitogen- and stress-activated protein kinases and induces IκBα degradation. Bone marrow-derived dendritic cells of C3H/HeJ and more pronounced C3H/HeJ/1 mice fail to respond to Gp96. Interestingly, activation of bone marrow-derived dendritic cells depends on endocytosis of Gp96 molecules. Our results provide, for the first time, the molecular basis for understanding the Gp96-mediated activation of antigen-presenting cells by describing the simultaneous stimulation of the innate and adaptive immune system. This feature explains the remarkable ability of Gp96 to induce specific immune responses against tumors and pathogens.

The immunogenic potential of heat shock proteins (HSPs) is a well-established phenomenon first observed by Srivastava during the immunotherapy of mouse tumors (reviewed in Ref. 1). Specificity of the immune response is based on peptides that associate with HSPs as a consequence of their function as molecular chaperones (2, 3). Tumor-specific protection is mediated by CD8+ T cells as shown by in vivo cell depletion studies (4) and by the ability to generate cytotoxic T lymphocyte cell lines specific for a variety of antigens from mice immunized with HSP molecules (5–7). The HSPs that mediated this effect include the cytosolic HSP70 and HSP90 and the endoplasmic reticulum-resident chaperins calreticulin and Gp96 (8, 9). Furthermore, Gp96 molecules have been shown to induce cytotoxic T lymphocyte cross-premising against viral and minor histocompatibility antigens, supporting the hypothesis that Gp96 molecules are associated with a large repertoire of peptides not influenced by the cellular major histocompatibility complex expression (6, 7). HSP molecules can also provide an immunogenic context to synthetic peptides complexed to HSP70 or Gp96 molecules in vitro (10, 11). Because of this, HSP molecules have been called adjuvants of mammalian origin (12). Recently, progress has been made in understanding the mechanisms that contribute to the efficient induction of immune responses against HSP-associated peptides.

A receptor responsible for the uptake of HSP-peptide complexes has been identified as the α2-macroglobulin receptor CD91, expressed on professional antigen-presenting cells (APCs) (9, 13). Only receptor-mediated endocytosis has been shown to result in the representation of HSP-associated peptides by MHC class I molecules, thus explaining the high efficiency of this process (14). However, the ideal adjuvant should not only target the antigen to professional APCs, it should also induce APC activation to provide the proper costimuli required for efficient induction of the immune response. For HSPs, especially HSP70, HSP90, and Gp96, this ability has been demonstrated recently. The exposure of macrophages or DCs to HSPs resulted in up-regulation of major histocompatibility complex class II and costimulatory molecules and in tumor necrosis factor α and IL-12 secretion (15–17). The contribution of this mechanism in situations of physiological relevance is supported by the observation that necrotic but not apoptotic cell death leads to the release of HSPs (17, 18), thus activating the innate arm of the immune system to attract cells equipped with antigen-specific receptors.

The molecular basis for this process, however, has not been understood thus far. Studies investigating the stimulatory effect of HSP60 on epithelial cells and that of HSP60 and HSP70 on macrophages demonstrated the involvement of CD14 molecules, suggesting the participation of Toll-like receptors (TLRs) (19). In Drosophila, Toll participates, in addition to the induction (coordination) of dorsal-ventral patterning during embryogenesis, in the defense against fungi by the induction of drosomycin secretion as an early form of innate immune response.
against infection (20). In mammals, TLRs are involved in the response to pathogens by the recognition of so-called pathogen-associated molecular patterns. These include lipopolysaccharide (LPS), peptidoglycans, and lipoproteins and bacterial CpG DNA, which are recognized by TLR4, TLR2, and TLR9, respectively (21, 22). The TLR signaling pathway shares most components with the IL-1 receptor signaling pathway responsible for activation of the innate immune system (21).

Recently, HSPs have been linked to TLRs by the observation that HSP60 failed to activate TLR4-defective macrophages from C3H/HeJ mice (23). Subsequently, it was shown that genetic complementation of nonresponder cells with TLR4 or TLR2 restores responsiveness (gain of function) to HSP60, whereas TLR2−/− or TLR4-deficient cells exhibit a “loss of function.” Surprisingly, macrophage activation was equally well induced by bacterial and endogenous mammalian HSP60. Thus, the presence of molecular patterns that interact with members of the TLR family is not limited to pathogen-derived molecules. Because of this, HSP60 has been proposed to serve as a danger signal for the innate immune system (19). HSP60 is not associated with antigenic peptides and is found much earlier in phylogeny, as are the TLR and IL-1 receptor families. Therefore, it remains unclear whether the HSP/TLR pathway is used solely by the innate immune system to fight pathogens by unspecific mechanisms or whether HSPs with peptide binding ability, such as Gp96, also use this mechanism to link nonspecific immunostimulatory capacities with their specific, peptide-based features with the activation of the adaptive immune system.

In this study, we decided to analyze the functional consequences of the interaction of Gp96 with members of the TLR family.

**EXPERIMENTAL PROCEDURES**

**Reagents, Antibodies, and Plasmin—Gp96 and FITC-labeled Gp96 were provided by Immatics Biotechnologies (Tübingen, Germany). LPS from *Salmonella minnesota* RE 595, monodansylcadaverine (MDC), and anisomycin were purchased from Sigma-Aldrich. Phosphothioate CpG oligonucleotide 1668 (TCCATCACGTTCCTGATGC) was synthesized by Hopers.**

**Antibodies to ERK1/2 were obtained from Upstate Biotechnology (Lake Placid, NY), and other antibodies used in cell signaling studies, including anti-phospho-JNK1/2 (Thr183/Tyr185), anti-JNK1/2, anti-phospho-p38 (Thr180/Tyr182), anti-p38, and anti-Rel-a, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-STAT1 (Tyr705), and anti-STAT1, were from New England Biolabs (Frankfurt, Germany). Antibodies for fluorescence-activated cell-sorting analysis were purchased from BD Pharmingen.**

The generation of human dendritic cells—Human dendritic cells were prepared from freshly drawn blood from healthy donors. Peripheral blood mononuclear cells were isolated using a Ficoll density gradient (Lymphoprep; Nycomed, Oslo, Norway). The obtained cells were washed twice with phosphate-buffered saline and resuspended in X-Vivo 15 medium (Bio-Whittaker, Walkersville, MD) supplemented with 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Peripheral blood mononuclear cells were plated at a density of 20 × 10^6 cells/well. After 2 h at 37°C, nonadherent cells were removed by washing with phosphate-buffered saline. Adherent monocytes were cultured for 6 days in medium supplemented with 1% (v/v) human serum (Peel-Freeze, Brown Deer, WI), 1000 units/ml IL-4 (R&D Systems), and 20 ng/ml granulocyte macrophage colony-stimulating factor (Leukomax; Novartis Pharma GmbH, Nürnberg, Germany). The differentiation state of DCs was examined by flow cytometry. Only immature DCs that were CD11c+, CD14+, CD83+, and CD86+ were used for activation experiments. The fraction of activated DCs analyzed by CD83 expression was always <5%.

**Stimulation of DCs—Mouse DCs were stimulated by the addition of 1 μg Gp96, 1 μg heat-pretreated Gp96, or 2 μg/ml LPS. After 24 h, IL-12 (p40) and IL-10 concentrations in the supernatants were measured using standard sandwich enzyme-linked immunosorbent assay protocols. Antibodies and recombinant standards of both cytokines were obtained from BD Pharmingen.**

**Generation of Human Dendritic Cells—**Human DCs were prepared from freshly drawn blood from healthy donors. Peripheral blood mononuclear cells were isolated using a Ficoll density gradient (Lymphoprep; Nycomed, Oslo, Norway). The obtained cells were washed twice with phosphate-buffered saline and resuspended in X-Vivo 15 medium (Bio-Whittaker, Walkersville, MD) supplemented with 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Peripheral blood mononuclear cells were plated at a density of 20 × 10^6 cells/well. After 2 h at 37°C, nonadherent cells were removed by washing with phosphate-buffered saline. Adherent monocytes were cultured for 6 days in medium supplemented with 1% (v/v) human serum (Peel-Freeze, Brown Deer, WI), 1000 units/ml IL-4 (R&D Systems), and 20 ng/ml granulocyte macrophage colony-stimulating factor (Leukomax; Novartis Pharma GmbH, Nürnberg, Germany). The differentiation state of DCs was examined by flow cytometry. Only immature DCs that were CD11c+, CD14+, CD83+, and CD86+ were used for activation experiments. The fraction of activated DCs analyzed by CD83 expression was always <5%.

**Ligation of DCs—**Mouse DCs were stimulated by the addition of 1 μg Gp96, 1 μg heat-pretreated Gp96, or 2 μg/ml LPS. After 24 h, IL-12 (p40) and IL-10 concentrations in the supernatants were measured using standard sandwich enzyme-linked immunosorbent assay protocols. Antibodies and recombinant standards of both cytokines were obtained from BD Pharmingen. The capture antibody was bound to the enzyme-linked immunosorbent plate (MaxiSorbTM; Nunc, Roskilde, Denmark), the biotinylated detection antibody was revealed by streptavidin-conjugated horseradish peroxidase and 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma), and the assay was read at 405 nm. Furthermore, on day 2 after activation, expression of the costimulatory molecule CD86 was measured by flow cytometry (FACSCaliburTM, BD Pharmingen). Isotype controls of antibodies were used in all experiments to determine the appropriate background fluorescence.

For experiments in the presence of MDC, human monocyte-derived immature dendritic cells (day 7) were incubated with 0.5 or 1.0 μg/ml Gp96, 1.0 μg/ml Gp96 heat-inactivated at 95°C for 20 min, or 2 μg/ml LPS in 0.5% Me2SO at 250 μg/ml MDC for 16 h. Supernatants were assayed for tumor necrosis factor α and IL-12 by sandwich enzyme-linked immunosorbent assay as described above.

A. Schild and H. Schild, unpublished observation.
Confocal Microscopy—Human monocyte-derived dendritic cells (day 7) were seeded on cover slips. The DCs were precooled and incubated for 30 min on ice with Iscove’s modified Dulbecco’s medium containing 10% fetal calf serum and 100 μg/ml Gp96-FITC (“pulse”) in 0.5% Me2SO. The coverslips were washed twice and incubated in Iscove’s modified Dulbecco’s medium for 15 min at 37°C (“chase”). Fixation was done in methanol/acetone (ratio, 1:1) at 20°C. For confocal microscopy, a Zeiss LSM 510 laser scanning microscope was used. Thickness of the optical plane was adjusted by the pinhole to be 1 μm.

RESULTS

Gp96 Interacts with TLR2 and TLR4—Among the members of the TLR family, the ligands for TLR2, TLR4, and TLR9 have been studied in considerable detail. Interestingly, HSP60 has been shown to activate macrophages via TLR4 (23). Inspired by these observations, we investigated the potential of these TLRs to trigger Gp96-mediated APC activation (14, 17). For this purpose, we incubated 293T human embryonic kidney fibroblasts transiently transfected with different TLRs and the luciferase reporter driven by synthetic enhancer containing NF-κB binding consensus sites with Gp96 or LPS as a control.

As shown in Fig. 1, the expression of TLR2 and TLR4/MD2 conferred responsiveness to the Gp96 stimulus in a dose-dependent manner. Boiling of Gp96 abolished the induction of luciferase activity, thus demonstrating that possible endotoxin contaminations in the Gp96 preparation were not responsible for the observed effect. Likewise, the presence of polymyxin B (an LPS inhibitor) did not interfere with Gp96-mediated activation (data not shown).

TLR4-mediated Gp96 activation is dependent on the presence of MD2 because transfection with TLR4 or MD2 alone did not result in the induction of luciferase activity (stimulation indices between 1.2 and 1.5; data not shown).

Gp96 Mediates DC Activation via TLR4 and TLR2—To an-
analyze the contribution of TLR2 and TLR4 under more physiological situations, we studied the Gp96-mediated activation of bone marrow-derived DCs (BMDCs) in mice lacking functional TLR2 or TLR4 molecules or both. In the first set of experiments, we investigated the secretion of the pro-inflammatory cytokine IL-12 in response to Gp96, LPS, and CpG DNA. As shown in Fig. 2, BMDCs from C3H/HeN, TLR2/H11002/H11002, and TLR2 wild-type mice but not from C3H/HeJ (a TLR4-deficient mouse) and C3H/HeJ/TLR2/H11002/H11002 (TLR2/4-deficient) mice responded to the Gp96 stimulus by secretion of IL-12. CpG DNA induced IL-12 secretion in all cultures, and LPS-mediated activation was impaired in BMDCs from C3H/HeJ and C3H/HeJ/TLR2/H11002/H11002 mice, in line with previous reports. Again, boiled Gp96 did not induce any stimulation. The lack of TLR2 did not affect Gp96-mediated IL-12 secretion from BMDCs.

During Gp96-mediated DC activation, secretion of IL-12 is accompanied by up-regulation of the costimulatory molecule CD86 (14, 17). Therefore, CD86 expression in mice lacking TLR2 or TLR4 molecules was investigated. As observed for the secretion of IL-12, BMDCs from C3H/HeN and C3H/HeJ/TLR2/H11002/H11002 mice up-regulated CD86 molecules after a 48-h coculture with Gp96. Fig. 3a shows CD86 up-regulation for one C3H/HeN mouse and one C3H/HeJ mouse; Fig. 3b represents the mean values of CD86 up-regulation of three independent mice. This effect was again heat-sensitive. CpG DNA induced CD86 up-regulation on all BMDCs, whereas LPS was impaired in BMDCs from C3H/HeJ and TLR2/4-deficient mice. A minimal up-regulation of CD86 molecules in C3H/HeJ mice compared with TLR2/4-deficient mice was observed in three independent experiments and might be caused by the interaction of Gp96 with TLR2 as observed for the NF-κB-driven luciferase induction reported in Fig. 1. In TLR2-deficient mice, this effect can obviously be compensated by TLR4.

Gp96 Activates Classical Signaling Cascades—The interaction of Gp96 with TLR2 and TLR4 suggests that the activation of APCs involves the classical signaling cascades described for other TLR2 and TLR4 ligands (22, 25). To analyze this issue, the macrophage cell line RAW264.7 was incubated with different concentrations of Gp96 and probed for the phosphorylation of JNK1/2, stress-activated protein kinase (SAPK) p38, the mitogen-activated protein kinase (MAPK) ERK1/2, and the degradation of IκBα as an indication for the activation of the NF-κB pathway. Fig. 4 shows that all stress kinases tested were activated. The Gp96 effect was again heat-sensitive. CpG DNA was used as a positive control.

DC Activation Requires Endocytosis of Gp96—Because major histocompatibility complex class I-restricted representation...
of HSP70- as well as Gp96-associated peptides depends on receptor-mediated, clathrin-dependent endocytosis of these molecules and subsequent transport to multivesicular compartments (14, 26, 27), we investigated whether endocytosis of Gp96 might be a prerequisite for DC activation. MDC is an inhibitor of the membrane-bound transglutaminase and interferes with clathrin-mediated receptor trafficking as demonstrated for the α2-macroglobulin receptor, CD91 (28). Interestingly, CD91 has been shown to be responsible for Gp96 uptake and representation of the associated peptides (9, 13) after receptor-mediated endocytosis (14).

Indeed, MDC was found to inhibit the endocytosis of FITC-labeled Gp96 molecules by DCs (Fig. 5). The presence of 0.5% Me₂SO did not influence Gp96 uptake compared with medium control lacking Me₂SO (data not shown). Therefore, the accumulation of Gp96-FITC at the cell membrane is due to the effect of MDC.

The lack of Gp96 endocytosis in the presence of MDC is accompanied by the lack of tumor necrosis factor α secretion (Fig. 6). As a control, LPS-mediated DC activation is not inhibited by MDC. These results also argue against an endotoxin contamination in the Gp96 preparation being responsible for the observed effect. Similar results were obtained for the Gp96-induced secretion of IL-12 (data not shown).

We next analyzed the effect of MDC on the signal transduction pathways and found that it also interfered with the phosphorylation of JNK1/2 (Fig. 7). The inhibitory effect of MDC was dose-dependent (data not shown) and did not influence the phosphorylation of JNK1/2 and p38 as well as the mitogen-activated protein kinase ERK1/2 (Fig. 4). DC activation is inhibited by MDC, which interferes with receptor-mediated endocytosis (Figs. 5–7).

Despite the fact that Gp96 and LPS are very different molecules, they both mediate DC activation via TLR4 and TLR2. The nature of the pathogen-associated molecular pattern of HSPs that allows their specific interaction with TLR2 and TLR4 is unknown. We are currently investigating the possibility that Gp96 and microbial components interfere with each other in binding to both TLRs. However, regardless of their shared interaction with TLR2 and TLR4 molecules, several differences in their mode of action are apparent. The most striking observation is that the Gp96-mediated DC activation strictly depends on the endocytosis of Gp96, whereas LPS-mediated DC activation does not require endocytosis and thus functions in the presence of the endocytosis inhibitor MDC. As a consequence, Gp96- but not LPS-mediated secretion of cyto-
kines is impaired in the presence of MDC (Fig. 6). This, together with the observed sensitivity of Gp96-mediated DC activation to heat denaturation, also demonstrates that an endotoxin contamination in the Gp96 preparation does not account for the observed effects.

This is further supported by the observation that cells expressing Gp96 molecules targeted to their surface induce efficient DC maturation upon cell-to-cell contact (35). Internalization of TLR2 during the activation of macrophages has been observed previously (29). In this series of experiments, TLR2 was found to accumulate in phagosomes of macrophages activated with the yeast cell wall particle zymosan. Whether or not endocytosis was a prerequisite for activation was not investigated. Our results obtained with Gp96 as an agonist for TLR2 and TLR4 suggest this to be the case.

The importance of HSP endocytosis for macrophage activation has very recently been reported for the HSP60/TLR2/4-mediated activation of macrophages (30). Here we observe the same effect for the new TLR2 and TLR4 ligand, Gp96, and extent. Our finding parallels the need for Gp96 endocytosis during receptor-mediated uptake and representation of Gp96-associated peptides (14, 26) that is mediated through the interaction of Gp96 with CD91, the receptor for α2-macroglobulin (9, 13). Interestingly, MDC had first been described as an inhibitor of endocytosis of the α2-macroglobulin receptor (28).

An interesting scenario can be postulated from the above findings: similar to the proposed requirement of LPS-mediated activation for CD14 and LPS-binding protein on the cell surface (reviewed in Ref. 31), Gp96-mediated DC activation might depend on the presence of CD91 molecules that endocytose bound Gp96 molecules and subsequently mediate their transport to endocytic vesicles, as described previously (26). This process will increase the local concentration of Gp96, now able to trigger signaling through TLR2 and TLR4 present in these vesicles (32) by the recruitment of cytosolic MyD88 to the outer membrane of endocytic vesicles.

The contribution of TLR2 to Gp96-mediated DC activation is not clear. Whereas transfection of TLR2 induces NF-κB-driven luciferase activity (Fig. 1), TLR2−/− mice show normal Gp96-mediated DC activation profiles (Figs. 2 and 3b). On the other hand, Gp96-mediated DC activation in C3H/HeJ mice is always stronger than that in TLR2−/−/TLR4-deficient mice, which are not able to respond to a Gp96 stimulus at all. This observation suggests a minor contribution of TLR2. In TLR2−/− mice, the lack of TLR2 signaling can apparently be completely compensated by TLR4, but TLR2 can induce only minimal activation when TLR4 is not functional, as observed for BMDCs from C3H/HeJ mice. One explanation could be an imbalance in the expression of these receptors on the cell surface (33).

Thus far, TLRs have been described as sensors for pathogen-associated molecular patterns crucial for the initiation of an innate immune response. These mechanisms were developed long before the adaptive immune system. One of the newest additions to the list of TLR ligands identified is HSP60. Interestingly, not only bacterial but also human HSP60 cross-reacts with TLRs (30). Our results now demonstrate that the exclusive association of TLRs with pathogen-associated molecular patterns is obsolete. Gp96 is not expressed in bacteria or fungi.
and provides the first example of a non-pathogen-derived ligand of TLRs. More importantly, our results show for the first time how the innate and adaptive immune system can be stimulated simultaneously by the same molecule that is released under physiological situations from necrotic cells (17, 18). The importance of these TLR-mediated stimuli for the induction of T helper type 1-dominated immune responses has been observed recently using MyD88−/− mice (34).

Gp96 has kept the ability (probably HSP60-derived) to stimulate APCs nonspecifically via TLRs but has added a new function: to act as a carrier for antigenic peptides and to promote receptor-mediated uptake by professional APCs (14, 26). The unique combination of both features now allows the major histocompatibility complex-restricted presentation of antigenic peptides to cells of the adaptive immune system in an immunostimulatory context and enables DCs to act as coordinators of innate and adaptive immune responses. Being able to understand these mechanisms will make it possible to interfere with the HSP-mediated activation of APCs and to rationally modulate immune responses toward either immunity or tolerance.

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