The Journal of Immunology

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J Immunol 1999; 162:3757-3760; ;
http://www.jimmunol.org/content/162/7/3757

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cutting Edge: Receptor-Mediated Endocytosis of Heat Shock Proteins by Professional Antigen-Presenting Cells[1]

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Immunization with heat shock proteins (HSPs) induces Ag-specific CTL responses. The specificity of the immune response is based on peptides associated with HSPs. To investigate how exogenous HSP/peptide complexes gain access to the MHC class I-restricted Ag presentation pathway, we incubated the monocytic cell line P388D1 and the dendritic cell line D2SC/1 with gold-labeled HSPs gp96 and HSC70. We show that HSPs bind specifically to the surface of these APCs and are internalized spontaneously by receptor-mediated endocytosis, demonstrating the existence of specific receptors for HSPs on these cells. In addition, we observe colocalization of internalized HSPs and surface MHC class I molecules in early and late endosomal structures. These findings provide possible explanations for the immunogenicity of HSP/peptide complexes and for the transfer of HSP-associated peptides onto MHC class I molecules. ***The Journal of Immunology,*** **1999, 162: 3757–3760.**

Heat shock proteins (HSPs)[4] have been shown to induce CTL responses against Ags expressed in cells from which they have been isolated (reviewed in ref. 1). The specificity of this immune response is based on peptides derived from endogenous proteins and associated with the HSP molecules (2, 3). This property allows the induction of CTL responses via HSP immunization, without the need to characterize the corresponding Ag, and thus prompts the development of new strategies for immunotherapy of cancer and infectious diseases.

Not only do HSPs carry endogenous peptides but they also allow efficient presentation of their associated peptides to T cells. Indeed, recently it was demonstrated that as little as 1–2 nanograms of peptides complexed to gp96 were able to induce a peptide-specific CTL response in vivo (4). To explain this efficient introduction of HSP-associated peptides into the MHC class I-restricted Ag presentation pathway, it was postulated that HSP/peptide complexes are taken up by professional APCs, such as macrophages or dendritic cells (DCs) that are specialized in the induction of CTL responses. The involvement of these types of cells was suggested by experiments based on in vivo depletion of phagocytotic cells (5) and stimulation of CTLs by macrophages pulsed with gp96/peptide complexes in vitro (6). The high efficiency and selectivity of this process led to the postulation of HSP-specific receptors on professional APCs that take up HSP/peptide complexes and shuttle them into the MHC class I-restricted Ag presentation pathway (7).

We investigated in this study the involvement of a receptor-mediated endocytosis (RME) of HSPs by professional APCs using the lymphoid macrophage line P388D1 and the DC line D2SC/1.

Materials and Methods

Cell lines and Abs

P388D1 and IGELa2 mouse cell lines, obtained from the American Type Culture Collection (ATCC; Manassas, VA), were cultured in RPMI 1640. D2SC/1 cells (8), kindly provided by Dr. Paola Paglia, were cultured in Iscove's modified Dulbecco's medium. All tissue culture media were supplemented with 10% FCS, 0.3 mg/ml L-glutamin, 100 U/ml penicillin/streptomycin, and 2 μl/ml 2-ME. Abs to gp96 (SPA-850) and to HSC70 (SPA-815) were obtained from StressGen Biotechnologies (Victoria, BC, Canada). The anti-CD32 (IV.3) Fab fragments were purchased from Medarex (Annandale, NJ). The anti-H2-K^d (K9-18, obtained from ATCC) F(ab′)$_2$ fragments were prepared by a complete digestion of 1 mg K9-18 with 10 U pepsin in 0.2 M sodium acetate, pH 5.0.

Purification of stress proteins

gp96 and HSC70 were purified from IGELa2 cells as described (9, 10). The approximate concentrations were determined by measuring the OD at 280 nm using an extinction coefficient of 1.0.

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Received for publication December 2, 1998. Accepted for publication January 21, 1999.

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[1] This work was supported by grants from the Deutsche Forschungsgemeinschaft (Leibnizprogram to H.G.R. (Ra369/4-1), Sonderforschungsbereich 510, C1 to H.S.), the European Union (Biotech 95-1627 and Biomed 95-0263), by the Etablissement de Transfusion Sanguine de Strasbourg, by the Institut National de la Santé et de la Recherche Médicale (CJF 94-03) and by Grant FORTS 96 from the Agence Francaise du Sang (Paris, France).

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[4] Abbreviations used in this paper: HSP, heat shock protein; DC, dendritic cell; HSC70-Au, gold-labeled HSC70; gp96-Au, gold-labeled gp96; RME, receptor-mediated endocytosis.

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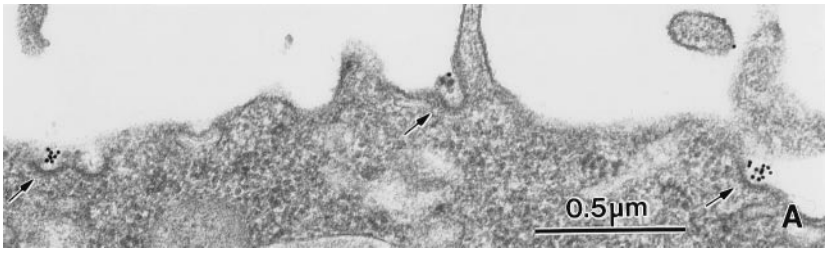

FIGURE 1. Spontaneous localization of gp96-Au in coated pits at 4°C. Precooled P388D1 cells were incubated for 60 min at 4°C in the presence of gp96-Au10. Cells were then fixed at 4°C and prepared for electron microscopy. Note the clustering of gold-conjugated gp96 in three coated pits (arrows).

Internalization experiments

The labeling of proteins with gold particles was performed as previously reported (11). The stress proteins and the IV.3 Fab fragments were conjugated to 10-nm gold particles (Goldsols EM-10 nm; Aurion, Wageningen, The Netherlands), whereas the $F(ab')_2$ fragments of the K9-18 mAb were labeled with 6-nm gold particles (Goldsols EM-6 nm; Aurion). BSA conjugated to 10-nm gold particles (BSA-Au10) was directly purchased by Aurion. For each experiment, 5×10^5 P388D1 or D2SC/1 cells were cooled to 4°C, then incubated with 1 μg of gold-labeled stress protein or control protein (BSA-Au10 or IV.3 Fab-Au10). The cells were either kept at 4°C or warmed to 37°C for 5 min and then fixed for electron microscopy. For the competition experiments, 5×10^5 precooled D2SC/1 cells were incubated for 30 min at 20°C with 1 μg gp96-Au10 alone or 1 μg gp96-Au10 and a 500-fold excess of the unlabeled competitor protein (yeast mannan was used at 0.6 mg/ml). The colocalization experiment was performed by adding to the cells at the same time an equal amount of gp96-Au10 and K9-18 $F(ab')_2$-Au6. Finally, in pulse-chase experiments to label the late endocytic compartments, 5×10^5 D2SC/1 cells were incubated for 10 min at 37°C in the presence of the same amount of gp96-Au10 and K9-18 $F(ab')_2$-Au6, washed extensively, and chased for 20 min at 37°C.

Preparation of cells for electron microscopy

Depending on the experiments, the cells were fixed at 4°C, 20°C, or 37°C with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 2% sucrose (305 mOsm, pH 7.3), washed, and postfixed with 1% osmium tetroxide (Merck, Darmstadt, Germany) in 0.1 M sodium cacodylate buffer. After dehydration, the cells were embedded in Epon (Electron Microscopy Sciences, Euromedex, Strasbourg, France). Ultrathin sections, stained with lead citrate and uranylacetate, were examined under a Philips CM 120 BioTwin electron microscope (120 kV).

Results and Discussion

Receptor-mediated endocytosis of gp96

To investigate the endocytic pathway of gp96, we incubated the macrophage cell line P388D1 with gp96-Au10. At 4°C, the gp96-Au10 particles were located in clathrin-coated pits (Fig. 1), suggesting the existence of a receptor for this HSP. The binding of gp96-Au10 to clathrin-coated pits at 4°C rules out the possibility that multivalent gp96-Au10 ligands are internalized by the cross-linking of the putative HSP receptor. We thus conclude that there exists apparently a spontaneous and continous internalization of the gp96 receptor. No staining was observed when the P388D1 cells were incubated in the same conditions with gold-labeled control proteins, BSA-Au10 or IV.3 Fab-Au10 (data not shown). When the cells were further incubated at 37°C, the electron micrographs show a distribution of the gold particles not only in clathrin-coated pits (Fig. 2, *A* and *B*), but also in coated vesicles (Fig. 2*C*) and in endosomal-like compartments (Fig. 2*D*). Freshly isolated mouse dendritic epidermal Langerhans cells but not keratinocytes showed the same results (data not shown), as well as the DC line D2SC/1 (see Table I and Fig. 4). Taken together, these experiments suggest that a RME is involved in the uptake of gp96 by APCs and that receptor-endocytosed gp96 reaches endosomal structures.

Receptor-mediated endocytosis of HSC70

To determine the route of endocytosis used by the heat shock protein HSC70, we examined ultrathin sections of the P388D1 cells pulsed with HSC70-Au10. The gold particles were also found associated to clathrin-coated pits at the cell surface when the cells were incubated with HSC70-Au10 either at 4°C (data not shown) or for 5 min at 37°C (Fig. 3, *A* and *B*). Likewise, after incubation at 37°C gold particles were also distributed inside the cells in clathrin-coated vesicles (Fig. 3, *B* and *C*) and in endosomal-like structures (Fig. 3*D*). Compared with gp96-Au10, the number of clathrin-coated structures observed with HSC70-Au10 was about 5- to 10-fold lower. Nevertheless, it seems that not only gp96, but also HSC70 molecules are internalized by a RME involving coated structures. In line with this, Srivastava and colleagues (12) recently observed the binding of HSPs to freshly isolated peritoneal exudate cells.

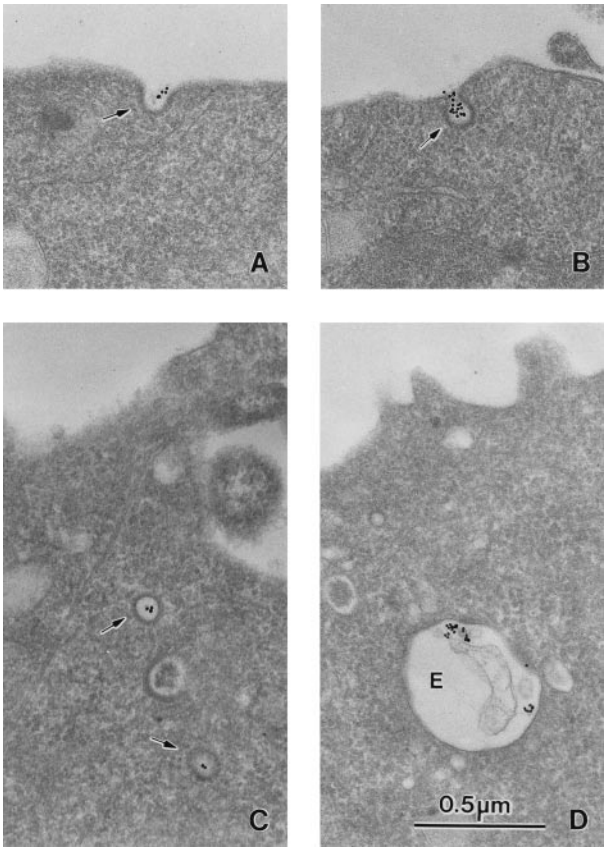

FIGURE 2. Internalization by receptor-mediated endocytosis of gp96-Au molecules. Precooled P388D1 cells were incubated in the presence of gp96-Au10 for 60 min at 4°C, followed by 5 min at 37°C. Fixation was performed at 37°C. *A* and *B*, gp96-Au associated with coated pits (arrows). *C*, The gp96-Au10 is apparently located in two coated vesicles (arrows). *D*, gp96-Au10 labels an endosomal structure (E). Note that while some gold particles label the endosomal membrane, the majority of the gp96-Au is located arround the intraluminal vesicles as suggested by visualization of the endosome under different angles (data not shown). *A–C* are at the same magnification as *D*.

Table I. *Distribution of gp96-Au particles in preendosomal structures*[a]

Competitor	% Au Particles in Coated Structures	% Au Particles in Noncoated Structures
None	32	68
BSA	42	58
gp96	8	92
HSC70	33	67

[a] D2SC/1 cells were incubated with gp96-Au alone or with gp96-Au and a 500-fold molar excess of unlabeled competitor for 60 min at 4°C followed by 30 min at 20°C. Cells were then fixed at 20°C and prepared for electron microscopy. At 20°C, prelysosomal late endosomes no longer fuse with newly formed endosomes. Indeed, at this relatively low temperature, the number of coated pits per cell section was the highest (up to 11) and therefore the reduction observed is more reliable. We did not attempt to compete HSC70-Au10 molecules with unlabeled HSC70 or gp96 molecules because the low number of coated pits observed with HSC70-Au10 did not allow a reliable quantitation of the inhibition experiments. For each experiment, 30 cells were randomly selected and the gold particles in preendosomal structures were counted (about 700 Au-particles for each condition) and converted to % Au particles in coated or noncoated structures.

Specificity of gp96 internalization through its receptor

Competition experiments were performed with D2SC/1 cells to examine the specificity of the RME of gp96 molecules. In the absence of competitor, 32% of the gold particles localized to coated pits/vesicles and 68% to noncoated structures (Table I). In contrast to the competition with BSA and HSC70, only an excess of nonlabeled gp96 was able to change significantly the distribution of the gold particles; the majority (92%) of the gp96-Au was present in uncoated regions/compartments and only 8% remained in clathrin-coated structures (Table I). As expected, the number of gold particles found in noncoated structures, that are involved in nonreceptor-mediated, unspecific endocytosis could not be reduced, indicating that the binding of gp96 molecules to their receptor is a specific interaction. Moreover, the finding that HSC70 is unable to compete with the gp96-Au staining might indicate that two different receptors exist, one specific for gp96, the other for HSC70. However, an alternative explanation could be that the affinity of the receptor differs dramatically between gp96 and HSC70 molecules and that HSC70 cannot be used at concentrations high enough to compete with gp96. To rule out the possibility that the glycoprotein gp96 uses its carbohydrate structures to enter the cell via the mannose receptor, we performed an additional competition experiment using mannan as a competitor. No inhibition effect was observed using mannan at a concentration of 0.6 mg/ml (data not shown), whereas 0.3 mg/ml mannan was shown to inhibit completely the uptake of FITC-dextran by the mannose receptor (13). These data indicate that the receptor used by gp96 is probably not only different from the mannose receptor, but also different from the receptor used by the non-glycosylated HSC70.

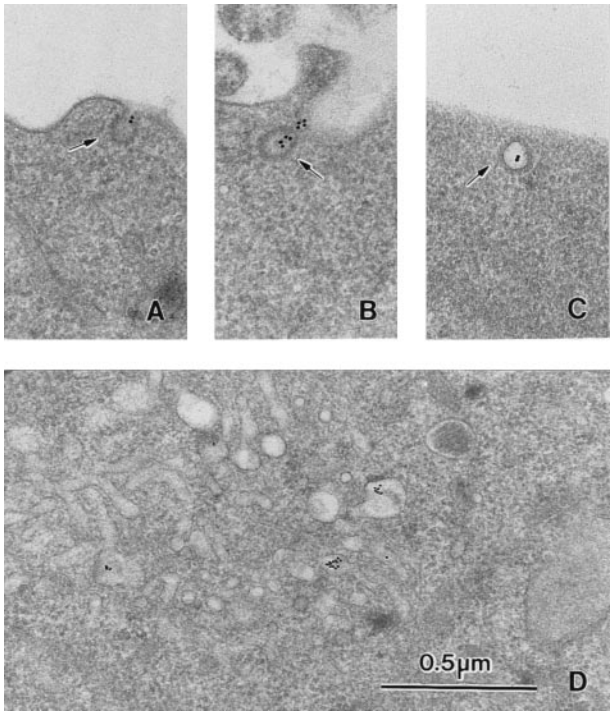

FIGURE 3. Internalization by receptor-mediated endocytosis of HSC70-Au molecules. Precooled P388D1 cells were incubated with HSC70-Au10 and treated as in Fig. 2. *A–C*, HSC70-Au10 associated with coated structures (arrows). HSC70-Au10 is also visualized in structures belonging to the early tubulo-vesicular endosomal system (*D*). *A–C* are at the same magnification as *D*.

Colocalization of gp96 and anti-H2-K^d in clathrin-coated and endosomal structures

To gain information about the mechanism by which gp96-associated peptides can access to the MHC class I pathway, we investigated the respective localization of MHC class I molecules and gp96 molecules internalized by their receptor. We first checked the fate of MHC class I molecules in D2SC/1 cells by repeating internalization experiments using gold-labeled F(ab′)$_2$ fragments of the mAb K9-18 specific for the H2-K^d molecules. It appeared that D2SC/1 cells internalize spontaneously their H2-K^d molecules via coated pits, coated vesicles (6-nm gold particles on Fig. 4) and endosomal structures as reported before for MHC class I molecules expressed on other DC lines (14, 15). Next, we incubated the DCs with gp96-Au10 and with K9-18 F(ab′)$_2$-Au6 at the same time. Our results show that after a 5 min incubation at 37°C, both the 10-nm and the 6-nm gold particles could be found together at the cell surface in clathrin-coated pits (Fig. 4*A*) as well as inside the cells in coated vesicles (Fig. 4*B*) and in early endosomal structures (data not shown). At 20-min chase, multivesicular compartments were reached by gp96-Au10 and K9-18 F(ab′)2-Au6, the colabeling being mainly located arround the internal vesicles (Fig. 4*C*). These findings indicate that MHC class I molecules and receptor-bound gp96 molecules can internalize together from the cell surface and finally colocalize in early and late multivesicular endosomal structures. This observation suggests that peptides might be exchanged between gp96 and MHC class I molecules in these compartments. The low pH in endosomal structures could favor the dissociation of peptides from gp96 molecules (D.A.-S., H.-G.R., and H.S., unpublished observation) and enzymatic activities might be required to generate final CTL epitopes from longer precursor peptides associated with gp96 molecules. In addition, since multivesicular compartments are rich in MHC class II molecules (16), the transport of gp96 through these compartments could explain the latest results of Matsutake and Srivastava (17), in which exogenous gp96/peptide complexes appear to be able to re-present their peptides through the MHC class II pathway and stimulate CD4$^+$ T cells. Whether or not MHC class I molecules are loaded with HSP-associated peptides in a TAP-independent fashion, as our studies suggest, remains to be demonstrated. The finding that Brefeldin A inhibits the presentation of gp96 associated peptides (6) is also in agreement with our observation, since this drug does not only disturb the transport of newly synthesized MHC class I molecules but also the traffic of vesicles in the endosomal pathway (18).

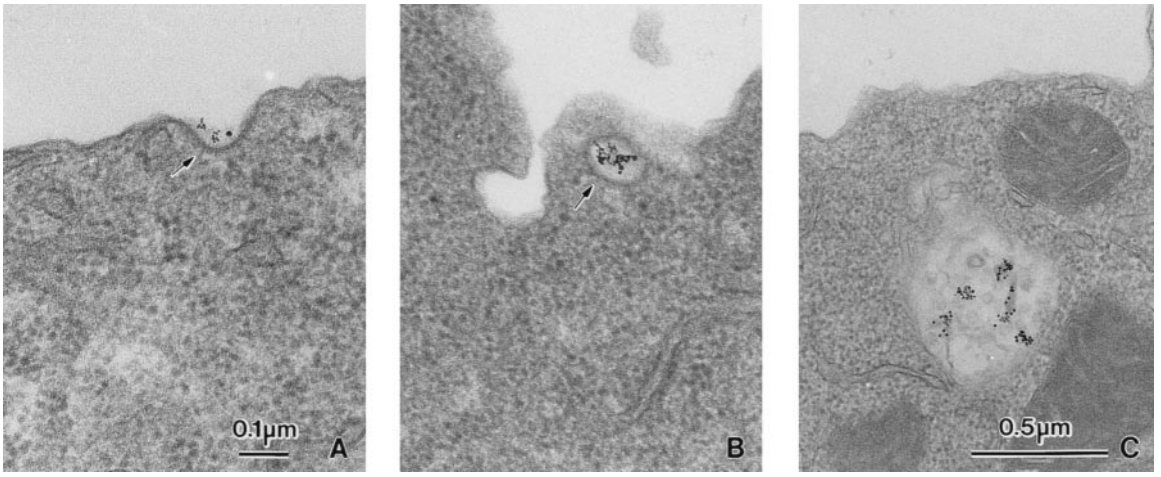

FIGURE 4. Cointernalization of gp96-Au10 and of the gold-labeled mAb anti-H2-K^d K9-18 F(ab′)$_2$-Au6. *A* and *B*, Precooled D2SC/1 cells were coincubated 60 min at 4°C, followed by 5 min at 37°C with the same amount of gp96-Au10 and K9-18 F(ab′)$_2$-Au6. Both gold-labeled molecules are present in the same coated structures (arrows). *C*, In a pulse-chase experiment D2SC/1 cells maintained at 37°C were incubated 10 min at 37°C in the presence of the same amount of gp96-Au10 and K9-18 F(ab′)$_2$-Au6, washed extensively, and chased for 20 min at 37°C before being fixed at 37°C for electron microscopy. Both gold-labeled molecules are present in the same multivesicular compartment, the colabeling being mainly located arround the internal vesicles. *B* is at the same magnification as *A*.

Conclusion

Our data provide the first evidence for a RME of HSPs. The presence of these receptors on APCs might explain the high immunogenic potential of HSPs in situations in which they are injected into mice or released from dying cells shuttling antigenic peptides to APCs as postulated earlier (7). Consistent with this hypothesis is the observation that high levels of HSP expression coincide with increased immunogenicity of tumor cells (19, 20). The HSP receptors allow the specific uptake of HSPs in a way that might be comparable to Ag uptake by the mannose receptor or the FcγR expressed on DCs (13, 21, 22). This receptor-mediated Ag presentation has been shown to be up to 10,000-fold more efficient than the presentation of peptides taken up by phagocytosis (21). If a similar scenario applies to the presentation of HSP-associated peptides, as suggested by our data, it is now possible to understand how as little as 1 ng of peptides complexed to gp96 is able to induce a CTL response in vivo (4). The next goal should now be the identification of the receptor(s) responsible for the internalization of HSPs and possible factors that modulate their expression on APCs. A further understanding of these processes would greatly enhance the application potential of HSPs in immunotherapy.

Acknowledgments

We thank Fabienne Proamer for technical assistance and Lynne Yakes for critical reading of the manuscript.

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