Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells

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Introduction

Investigations into the identity of the tumor-specific transplantation antigens of MethA sarcoma led to the discovery of gp96 (GRP94), the endoplasmic reticulum (ER) heat shock protein (Hsp) 90 molecular chaperone, as a potent tumor antigen (Srivastava et al., 1986; Srivastava and Maki, 1991). Subsequent studies indicated that a number of chaperones, including GRP170, Hsp110, Hsp90, Hsp70 and calreticulin (CRT) could elicit anti-tumor immune responses in both prophylactic and therapeutic immunotherapy settings (Udono and Srivastava, 1994; Tamura et al., 1997; Yamazaki et al., 1999; Srivastava and Amato, 2001). In the case of Hsp70, substantial experimental evidence has been provided to suggest that tumor-derived, Hsp70-associated peptides can function as tumor antigens (Udono and Srivastava, 1993; Milani et al., 2002; Noessner et al., 2002). For gp96, recent evidence indicates that gp96-mediated tumor suppression can occur through peptide-independent activation of anti-tumor immune responses (Baker-LePain et al., 2002). Indeed, the question of whether Hsps function as peptide binding proteins in vivo has recently come under question (Baker-LePain et al., 2003; Reits et al., 2003).

In a manner independent of bound peptides, gp96, Hsp70 and Hsp60 elicit dendritic cell maturation, activation and associated cytokine secretion (Asea et al., 2000, 2002; Binder et al., 2000a; Kol et al., 2000; Singh-Jasuja et al., 2000a; Vabulas et al., 2001, 2002; Zheng et al., 2001; Milani et al., 2002; Panjwani et al., 2002). At present, the APC receptors responsible for mediating the diverse interactions of Hsps with APC are poorly understood. Several recent studies have identified candidate receptors for the different Hsps. Initially, CD91 [LDL receptor-related protein (LRP)/α₂-macroglobulin (α₂M) receptor] was reported to be the unique receptor responsible for the re-presentation of peptides associated with gp96, Hsp70, Hsp90 and CRT (Binder et al., 2000b; Basu et al., 2001). However, it now appears unlikely that CD91 is the unique chaperone receptor; its expression does not positively correlate with chaperone binding capacity and both CD40 and the scavenger receptor LOX-1 have been demonstrated to function in Hsp70 internalization (Becker et al., 2002; Berwin et al., 2002a; Delsnè et al., 2002). Here we identify scavenger receptor class-A (SR-A) as a predominant endocytic receptor for the chaperones gp96 and CRT.

SR-A is expressed on macrophages and dendritic cells and was originally identified as a clearance receptor for acetylated low-density lipoprotein (AcLDL) (Kodama et al., 1988; Platt and Gordon, 1998). Subsequent work has demonstrated that SR-A serves multiple roles in innate immunity, including the receptor-mediated phagocytosis of bacteria and the internalization of lipopolysaccharide and lipoteichoic acid (Hampton et al., 1991; Peiser et al., 2003). Recent research has focused on the mechanism(s) by which these proteins elicit anti-tumor responses, with particular emphasis being placed on their interactions with antigen-presenting cells (APCs).

It is well established that complexes of Hsps and chaperone proteins with synthetic peptides can direct peptides into the MHC class-I antigen-presentation pathways of APCs (Suto and Srivastava, 1995; Singh-Jasuja et al., 2000b; Berwin et al., 2002b). By this process, chaperone–peptide complexes are thought to elicit tumor-directed CD8⁺ cytotoxic T lymphocyte (CTL) responses (Srivastava and Udono, 1994; 2000b; Tamura et al., 1997; Yamazaki et al., 1999; Srivastava and Amato, 2001). In the case of Hsp70, substantial experimental evidence has been provided to suggest that tumor-derived, Hsp70-associated peptides can function as tumor antigens (Udono and Srivastava, 1993; Milani et al., 2002; Noessner et al., 2002). For gp96, recent evidence indicates that gp96-mediated tumor suppression can occur through peptide-independent activation of anti-tumor immune responses (Baker-LePain et al., 2002). Indeed, the question of whether Hsps function as peptide binding proteins in vivo has recently come under question (Baker-LePain et al., 2003; Reits et al., 2003).

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In addition, SR-A performs a signaling role in cells, acting through G\textsubscript{i/o} (Whitman et al., 2000; Post et al., 2002) and the protein tyrosine kinase Lyn (Miki et al., 1996). We report that cell surface binding and uptake of gp96 and CRT into macrophages is efficiently blocked by the SR-A ligands fucoidin and carrageenan (Radsak et al., 2003). Using genetic models we demonstrate that ectopic expression of SR-A confers cell surface binding and uptake of gp96 and CRT, whereas genetic deletion of SR-A impairs gp96 and CRT binding and uptake. Data are also provided demonstrating that SR-A can direct bound ligands to an endosomal compartment previously shown to be involved in antigen re-presentation.

**Results**

**Identification of the gp96/CRT receptor**

Binding of gp96 to APC cell surface receptors is saturable, of high affinity and is necessary for the trafficking of chaperone-associated peptides into the MHC class-I presentation pathway (Binder et al., 2000b; Singh-Jasuja et al., 2000b; Berwin et al., 2002a,b). To investigate the mechanism of chaperone uptake into APC, gp96 and CRT were covalently labeled with fluorophores and their interactions with APC examined (Wassenberg et al., 1999; Berwin et al., 2002b). Both proteins were observed to bind to cell surface receptors of APC, and this binding was competed by the polyanionic SR-A ligands fucoidin (Figure 1A–C; Radsak et al., 2003) and carrageenan (data not shown), but not by the polyanion chondroitin sulfate (Figure 1A). Fucoidin-dependent inhibition of gp96 binding to both elicited peritoneal macrophages (Figure 1B) and RAW264.7 macrophages (data not shown) was dose-dependent, with half-maximal inhibition at ~4 µg/ml fucoidin; ~90% of the total cellular binding activity was fucoidin-sensitive (Figure 1B). Similarly, CRT internalization of Alexa-conjugated calreticulin was assayed in the presence of increasing concentrations of non-conjugated gp96 or calreticulin. The partial inhibition of CRT internalization observed with protein ligands is contrasted by that seen with fucoidin (filled triangles). (E) The binding of the CD91 ligand \(\alpha_2\)M to elicited peritoneal macrophages is not inhibited by fucoidin. Alexa-conjugated \(\alpha_2\)M binding to peritoneal macrophages was performed in the presence of a 10-fold molar excess of fucoidin, as in (A). Solid lines in (A), (C) and (E) represent autofluorescence.

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in macrophages (Binder, 2000). The fucoidin sensitivity of α2M binding to macrophages was thus examined and, as previously reported (Imber et al., 1982), no inhibition of α2M binding by fucoidin was observed (Figure 1E). These data indicate that gp96 and α2M are recognized by distinct receptors.

Fucoidin and carrageenan are ligands for several receptor families, including members of the scavenger receptor superfamily (Peiser and Gordon, 2001). To distinguish between candidate classes of scavenger receptors, we investigated the effects of endotoxin-elicited receptor up-regulation on gp96 and CRT binding to RAW264.7 cells. As has been reported previously, in RAW264.7 cells, lipopolysaccharide (LPS)-induced up-regulation of a fucoidin-binding receptor is consistent with the class-A, but not class-B, scavenger receptors (Fitzgerald et al., 2000; Baranova et al., 2002). Binding of CRT (Figure 2A) and gp96 (data not shown) increased ~3-fold (geometric mean fluorescence) upon endotoxin treatment of RAW264.7 cells. Significantly, the endotoxin-elicited increase in CRT binding capacity was fucoidin sensitive (Figure 2B).

**SR-A-mediated gp96 and CRT internalization**

Scavenger receptors bind and internalize a diverse array of ligands including modified LDL, fucoidin, apoptotic cells, bacterial LPS and lipoteichoic acid, and poly(I) (reviewed in Peiser and Gordon, 2001). Although structurally unrelated, these ligands share the common property of being polyanionic, as are gp96 and CRT (Krieger and Herz, 1994). Not all polyanions are SR ligands, however, as exemplified by chondroitin sulfate. To evaluate SR-A as a candidate receptor for gp96 and CRT, we used HEK 293 cells, which do not express SR-A. 293 cells were stably transfected with the SR-AII cDNA under an inducible tet promoter. These cells are referred to as HEK-SRAIItet (Post et al., 2002). It should be noted that SR-AII is a splicing variant of the gene that encodes SR-AI, SR-AII and SR-AIII; SR-AIII is not expressed at the cell surface; no functional difference between SR-AI and SR-AII has been reported (reviewed in Peiser and Gordon, 2001).

gp96 and CRT uptake was examined in uninduced-(absence of SR-A expression) and induced- (SR-A-expressing) HEK-SRAIItet cells, with competition by fucoidin used as a control for ligand specificity. We observed that SR-A expression markedly enhanced (>50-fold) gp96 (Figure 3A and D) and CRT (Figure 3B) internalization. No significant uptake of gp96 or CRT was observed in uninduced HEK-SRAIItet cells. As a positive control, an established SR-A ligand, AcLDL, was used in parallel (Figure 3C). SR-A-mediated uptake of both gp96 and CRT was abrogated by the addition of an ~5× molar excess of fucoidin (Figure 3A, B and D). gp96 uptake was both receptor expression- and ligand concentration-dependent (Figure 3E). The EC50 of fucoidin-sensitive gp96 uptake by induced (SR-A-expressing) HEK-SRAIItet cells was 22 ± 12 nM (SD, n = 3), indicative of highly efficient uptake and complementary to previously observed high affinity cell surface binding of gp96 to macrophages (Berwin et al., 2002a). To extend these findings to APC, we examined the effects of fucoidin on chaperone binding and uptake in elicited macrophages. Using an established ligand uptake protocol (Berwin et al., 2002b), we observed that the SR-A ligand fucoidin inhibited gp96 (Figure 3F), but not α2M uptake (Figure 3G).

Having established that ectopically expressed SR-A is capable of mediating the internalization of gp96 and CRT and that inhibitory ligands of SR-A compete for gp96 and CRT binding and uptake, we examined the contribution of endogenous SR-A to chaperone uptake in APC using the genetic model of elicited macrophages derived from SR-A knockout (SRA−/−) mice (C57/BL6 background) (Suzuki et al., 1997; Kunjathoor et al., 2002). SR-A−/− macrophages were impaired in their ability to bind both gp96 (Figure 4A) and CRT (Figure 4B) as compared to macrophages derived from C57/BL6 mice, exhibiting an ~50% reduction in binding. Concomitantly, the ability of fucoidin to compete binding of chaperones to SR-A−/− macrophages was decreased.

We then assessed whether the impairment of chaperone binding to SR-A−/− APC led to a deficiency in chaperone uptake. Fluorescently labeled gp96 (Figure 5A) or CRT (data not shown) were incubated with peritoneal macrophages at 37°C in the presence of varying fucoidin concentrations. Fucoidin competed ~80% of chaperone binding, with half-maximal inhibition observed at ~2 μg/ml fucoidin. Extending the binding studies, we then tested SR-A−/− macrophages for their ability to internalize chaperone proteins. SR-A−/− derived macro-
phages displayed ~50% of the CRT uptake activity observed in C57/BL6-derived macrophages (Figure 5B). Similar results were observed for gp96 (data not shown). These studies, utilizing genetic models, further indicate that SR-A functions in the recognition and internalization of gp96 and CRT.

**SR-A directs re-presentation of gp96-associated peptides**

FcγR mediates the uptake of IgG–antigen complexes, with the antigen subsequently cross-presented on cell surface MHC class-I molecules (Regnault et al., 1999; Guyre et al., 2001). Previously, we demonstrated that Fc receptors, and...
their accompanying cargo, are directed into a subset of early endosomes that are also accessed by receptor-internalized gp96 (Mellman, 1996; Regnault et al., 1999; Guyre et al., 2001; Berwin et al., 2002b). Existing evidence suggests that this compartment can participate in a process of antigen (peptide) exchange onto mature MHC class-I molecules (Kleijmeer et al., 2001; Berwin et al., 2002b). As would be predicted from results indicating that gp96 and CRT share a common endocytic receptor, CRT and gp96 co-localize upon uptake to a peripheral endosomal subpopulation (Figure 6A–C; Berwin et al., 2002b) and, correspondingly, CRT also co-localizes with FcγR upon uptake (Figure 6D–F). To test whether SR-A-mediated uptake is responsible for trafficking chaperones into the re-presentation pathway, we determined whether fucoidin would inhibit gp96-dependent peptide re-presentation. To this end, the K b ovalbumin peptide epitope SIINFEKL (Ova) was complexed with gp96 and re-presentation of this peptide on macrophage MHC class-I molecules assessed using the reporter T-cell hybridoma B3Z (Karttunen et al., 1992; Shastri and Gonzalez, 1993) and the K b/Ova-specific antibody 25-D1.16 Ab (Porgador et al., 1997). Fucoidin inhibited the re-presentation of the gp96-associated Ova 52 ± 14% (Figure 6H), while fucoidin did not inhibit the presentation of Ova peptide alone (Figure 6G and H). These data indicate that SR-A can direct gp96–peptide complexes into an MHC class-I re-presentation pathway.

Discussion

We report that the scavenger receptor SR-A mediates the recognition and internalization of gp96 and CRT in elicited murine macrophages, a murine macrophage cell line and human cells expressing SR-A under control of an inducible promoter. The identification of SR-A as an endocytic receptor for gp96 and CRT further expands the scope of scavenger receptor family protein function in heat shock/chaperone protein recognition and internalization.

Extending from the observations that CD91 expression did not positively correlate with gp96 cell surface binding or internalization, and that αM*, the primary physiological ligand for CD91, did not block gp96 cell surface binding or uptake, we proposed the existence of an alternative, CD91-independent pathway for gp96 internalization by APC (Berwin et al., 2002a). The data in the current report identify a primary role for SR-A in such a pathway, a finding of particular significance, as receptor-

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Fig. 4. SR-A−/− macrophages are deficient in gp96 and CRT binding. Elicited peritoneal macrophages from C57/BL6 and SR-A−/− (C57/BL6 background) mice were assayed for their ability to bind and internalize gp96 and CRT. Cell surface binding experiments were performed for 30 min at 4°C; TR–gp96 (A) TR–CRT (B). Binding of either chaperone to SR-A−/− macrophages was ~50% that observed for C57/BL6-derived macrophages. Solid lines represent autofluorescence.

Fig. 5. SR-A mediates gp96 and CRT uptake. (A) Fluorescently labeled gp96 was incubated with elicited peritoneal macrophages for 30 min, 37°C in the presence of the indicated concentrations of fucoidin. Subsequently cells were washed, fixed, and chaperone uptake assayed by FACS analysis. (B) SR-A−/− macrophages were tested for their ability to endocytose CRT, relative to wild-type (C57Bl/6-derived) macrophages. Analogous to the experiments performed in (A), chaperone internalization experiments were performed for 7 min at 37°C. SR-A−/− macrophages display ~50% of the receptor-mediated chaperone uptake of control macrophages. Similar results were observed for gp96.
mediated endocytosis is the mechanism by which gp96-associated antigens gain access to the cross-presentation pathway of APC (Singh-Jasuja et al., 2000b). In further support of this proposal, we observed that >90% of macrophage cell-surface gp96 binding, and >75% of gp96 uptake, can be blocked by the SR-A ligand fucoidin. In contrast, \( \alpha_2M \) binding to macrophages is not competed by fucoidin. Furthermore, and as determined in SR-A+/− macrophages, over half of the macrophage binding and endocytosis of gp96 and CRT are directly attributable to SR-A. Intriguingly, the data regarding fucoidin inhibition of gp96 and CRT binding and uptake corresponds well with the levels of inhibition observed for other SR-A ligands, including apoptotic cells (Platt et al., 1996) and *Escherichia coli* (Peiser et al., 2000). These data thus suggest that there exists on macrophages redundant SR-A binding sites.

**Fig. 6.** SR-A directs gp96 and CRT to an FcR+ endosomal compartment competent for the re-presentation of gp96-associated peptides. (A–F) Fluorescein-labeled gp96 (A and C) or IgG (D and F) and TR-CRT (B, E and F) were allowed to bind to the surface of macrophages on ice and the cells were subsequently washed to remove unbound ligand. Cells were then warmed to 37°C for 10 min, to allow ligand internalization, and the cells subsequently fixed and processed for confocal microscopy. Extensive co-localization (yellow) of receptor-internalized gp96 and IgG with CRT was observed (C,F). (G) 100 nM Ova peptide was incubated with C57/BL6-derived peritoneal macrophages in the presence or absence of 250 \( \mu \)g/ml fucoidin for 2 h. Surface presentation of the Ova peptide in the context of MHC class-I molecules was then assessed with the K\(^b\)-, Ova-specific monoclonal antibody 25-D1.16. (H) gp96-Ova peptide covalent complexes (50 \( \mu \)g/ml; ~250 nM gp96) or unconjugated OVA peptide (100 nM or 1 \( \mu \)M) were incubated with C57/BL6-derived peritoneal macrophages in the presence or absence of 250 \( \mu \)g/ml fucoidin for 30 min. The macrophages were then washed and incubated with B3Z reporter T-cells, which recognize Ova in the context of K\(^b\) MHC class-I molecules. B3Z activation was scored as β-galactosidase activity.
ligand (fucoidin) binding/uptake activity (with another fucoidin-binding receptor accounting for ~30% of the chaperone binding), or that a compensatory receptor(s) is upregulated upon SR-A inactivation. With regard to either scenario, it is known that the anti-SR-A antibody 2F8 will block adhesion of macrophages to tissue culture plastic (Fraser et al., 1993), yet SR-A+− macrophages retain the capacity to adhere.

Scavenger receptors can function in antigen cross-presentation (Abraham et al., 1995; Bansal et al., 1999), a finding that places them at the crossroads of the innate and the adaptive cellular immune systems. SR-A seemingly utilizes pattern recognition to bind ligands including apoptotic cells, bacteria, modified lipoproteins, and now resident molecular chaperones of the ER (Peiser and Gordon, 2001). We find that SR-A activity can initiate the process of re-presentation of gp96-associated peptide antigens, while a scavenger receptor on human dendritic cells, LOX-1, has recently been shown to traffic Hsp70-associated peptides into the re-presentation pathway of dendritic cells (Delneste et al., 2002). Delivery of peptides on vehicles such as antibodies and chaperones has previously been shown to be highly effective in raising CD8+ T-cell responses and, in some cases, protective and previously been shown to be highly effective in raising et al. dendritic cells (Delneste et al., 2001). We find that SR-A activity can initiate the process of re-presentation of gp96-associated peptide antigens, while a scavenger receptor on human dendritic cells, LOX-1, has recently been shown to traffic Hsp70-associated peptides into the re-presentation pathway of dendritic cells (Delneste et al., 2002). Delivery of peptides on vehicles such as antibodies and chaperones has previously been shown to be highly effective in raising CD8+ T-cell responses and, in some cases, protective and therapeautic anti-tumor responses (Blachere et al., 1997; Regnault et al., 1999; Castellino et al., 2000; Delneste et al., 2002). Indeed, targeting peptides into APCs via heat shock/chaperone proteins is much more efficacious in generating in vivo responses than using peptide alone (Blachere et al., 1997). Intriguingly, recent data suggest that peptides derived from the intracellular processing of the Hsps themselves may be of immunological significance; Michaelsson et al. (2002) report that a fragment of the Hsp60 signal peptide can be presented by the non-classical MHC class-I molecule HLA-E. Such complexes are not recognized by CD94/NKG2A inhibitory receptors, potentially resulting in the activation of natural killer cell activity. We speculate that peptides generated from other Hsps may render similar effects.

Linked to the ability of chaperones to stimulate the innate immune system is the capacity of chaperone receptors to activate cellular signal transduction pathways. Several reports have indicated chaperones can signal through cell surface Toll-like receptors (TLRs) and CD14, thereby stimulating cell activation and cytokine secretion (Asea et al., 2000, 2002; Vabalas et al., 2001, 2002). However, endotoxin contamination of Hsp preparations continues to be a concern when assessing Hsp-mediated activation of cells (Gao and Tsan, 2003; Reed et al., 2003), particularly where TLRs and CD14 function as shared signaling receptors for both endotoxin and chaperones. Indeed, recent data indicates that cell activation and cytokine production previously ascribed to Hsp70 and gp96 is likely to be due to contaminating endotoxin (Bausinger et al., 2002; Gao and Tsan, 2003; Reed et al., 2003); further experiments are clearly required to delineate the role(s) of endotoxin-free heat shock/chaperone proteins in APC signalling. SR-A has previously been documented to signal to intracellular kinases and phospholipases through G\(_{\text{i/o}}\) upon binding of another ligand, AcLDL (Hsu et al., 1998; Whitman et al., 2000; Post et al., 2002). Additionally, there is evidence that SR-A can signal through Lyn kinase, a molecule with which it has been co-immunoprecipitated (Miki et al., 1996). Intriguingly, Lyn kinase is involved in ligand-stimulated signaling from FC receptors (Kovarova et al., 2001; Strzelecka-Kliszak et al., 2002), which, as described in this report, can be colocalized intracellularly with SR-A ligands and function in antigen uptake and cross-presentation. Additionally, a recent report indicates that gp96 elicits ERK, but not NF-κB, activation in murine macrophages (Reed et al., 2003). However, further studies are required to determine whether SR-A also functions in gp96 signaling in APC. These studies are currently underway.

Heat shock/chaperone protein trafficking and signaling via SR-A may also be of relevance to processes other than tumor immunity. For example, atherosclerotic plaque progression is accompanied by the formation of necrotic lesions bearing a substantial accumulation of macrophages (reviewed in Schwartz et al., 1991). As it is established that necrotic cells release intact ER-derived chaperones (Basu et al., 2000; Berwin et al., 2001), it can now be considered that heat shock/chaperone proteins released from dying cells present in atherosclerotic lesions may recruit macrophages to the site of the lesion, via interactions with SR-A, and thereby contribute to the etiology of atherosclerosis.

In conclusion, we demonstrate a novel endocytic mechanism for the ER chaperones gp96 and CRT, via scavenger receptor SR-A. Other ligands of SR-A, including fucoidin, compete for the binding and uptake of these chaperones, indicating a common binding site. However, these competing ligands are not structurally similar, implicating pattern recognition as a determinant for binding. Ectopic expression of SR-A is sufficient to induce receptor-mediated endocytosis in HEK 293 cells, while studies with SR-A+− macrophages indicate that SR-A is necessary for wild-type levels of gp96/CRT binding and uptake. Finally, targeting antigens to SR-A via gp96 results in their cross-presentation on APC cell surface MHC class-I molecules. These data expand the role of SR-A as a receptor involved in both the innate and adaptive arms of the cellular immune system.

Materials and methods

Cell culture and transfection

Peritoneal macrophages were elicited in C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME or Charles River, Wilmington, MA) as described previously (Wassenberg et al., 1999; Berwin et al., 2002b). SR-A null mice (Suzuki et al., 1997; Kunjathoor et al., 2002) were backcrossed into the C57BL/6J background and were a generous gift of T.Kodama (Tokyo University) and M.W.Freeman (Massachusetts General Hospital, NHLBI Program in Genomics Applications). Mice were sacrificed 4–5 days post-injection and macrophages were obtained by peritoneal lavage. Macrophages were enriched by adherence selection for 1 h in complete DMEM, 10% FCS on either 12-well plates (Corning Glass Works, Corning, NY) for flow cytometry experiments, or 18 mm 1 glass coverslips (VWR Scientific, Inc., Media, PA) for laser scanning confocal microscopy studies. RAW264.7 macrophages were cultured according to published ATCC protocols. Human embryonic kidney (HEK 293) cells expressing the tetracycline response protein (Flp-In™T-REX™-293; Invitrogen; Carlsbad, CA) were maintained in DMEM containing penicillin (10 U/ml), streptomycin (10 μg/ml), 10% FBS (DMEM/FBS), and blasticidin (15 μg/ml) and zeocin (100 μg/ml). The cDNA encoding the type II murine scavenger receptor (provided by A.Daugherty, University of Kentucky) was subcloned into the pcDNA5/FRT/TO vector (Invitrogen) and cotransfected into HEK cells with the POG44 plasmid encoding the flipase gene using Mirus Trans-IT-T93 transfection reagent (Panvera, Madison, WI) according to the manufacturer’s protocol. Using
this expression system, SR-A is only expressed when an inducing agent (e.g., tetracycline) is added to the cell culture media (Post et al., 2002). Transfected cells (HEK-SRA<sup>+</sup>) were selected with hygromycin (100 μg/ml) and inducible expression confirmed by immunostaining adherent cells with monoclonal antibody 2F8 and western blotting of cell lysates with a guinea pig anti-SR-A antibody (Post et al., 2002).

**Antibodies and proteins**

gp96 and CRT were purified by the method of Wearsch and Nicchitta (1996). Texas Red (TR)- and fluorescein (Fl)-succinimidyl esters, DiOC<sub>6</sub> and AlexaFluors were obtained from Molecular Probes (Portland, OR). Protein labeling with the succinimidyl ester conjugates was performed according to manufacturer’s protocols. 2F8 (anti-SR-A) was purchased from Serotec (Oxford, UK). Human α<sub>M</sub> was purified and labeled as previously described (Gron and Pizzo, 1998; Berwin et al., 2002a).

Fucoidin, carrageenan, PTX and chondroitin sulfate were obtained from Sigma (St Louis, MO). 25-D1.16 Ab (Porgador et al., 1997) was the generous gift of Dr J.Yewdell (NIAID, NIH).

**Detection of fluorescent ligand association**

Receptor-mediated- and fluid phase-uptake studies were performed as described previously (Wassenberg et al., 1999; Berwin et al., 2002a,b).

Fluid phase uptake of protein was accomplished by placing cells in 37°C medium containing the labeled protein. Cell surface receptor binding was achieved by incubating the cells at 4°C with the ligand, followed by washing and fixation. Cells were removed from 12-well plates with a cell scraper (Costar Corporation, Cambridge, MA) followed by analysis for fluorescence by flow cytometry (Becton Dickinson, San Jose, CA).

Further analysis was performed using Cell Quest (Becton Dickinson, San Jose, CA). HEK-SRA<sup>+</sup> cells were incubated for 24 h with tetracycline (0.5 μM) to induce SR-A expression. To assess SR-A-mediated uptake of chaperone proteins, cells were pre-incubated for 2 h in serum-free DMEM and then incubated for 1 or 2 h with fluorescent ligand. Where indicated, the SR-A antagonist fucoidin (75 μg/ml) was added 5 min prior to addition of fluorescent ligand. Unbound ligand was removed by washing cells with PBS, cells suspended by trypsinization, and associated fluorescence determined by flow cytometry. EC<sub>50</sub> of gp96 uptake by HEK-SRA<sup>+</sup> cells was determined by the aforementioned uptake assay, using 75 μg/ml fucoidin in parallel assays to determine non-specific binding and uptake. The EC<sub>50</sub> was determined from a least squares fit of the data to one phase of a hyperbolic curve.

**Fluorescent staining and confocal imaging**

HEK-SRA<sup>+</sup> cells were plated (50 000 cells/well) on two-chambered LAB-TEK slides (Nalge Nunc International, Naperville, IL). To facilitate adhesion of uninduced cells, the slides were treated with BD Cell-Tak (BD Biosciences, Bedford, MA) according to manufacturers’ adsorption protocol. SR-A expression was induced by addition of tetracycline (0.05 μM). After 16 h, the media was removed and cells equilibrated in serum-free DMEM with 0.2% BSA for 2 h. Cells were gently washed twice for 2 min with warm phenol red-free DMEM, and, where indicated, incubated with fucoidin (75 μg/ml) for 10 min before addition of fluorescent ligand. Fluorescent ligands were incubated with cells at 37°C for 1 h. Ligand association was terminated by washing cells twice with ice-cold PBS. Cells were then mounted in the embedding medium Mowiol containing 1% n-propyl gallate. Images were digitally captured using a Zeiss LSM 410 confocal microscope (Thornwood, NY). For confocal microscopy analysis of macrophages the cells were incubated with ligands as described in the text. The cells were then washed and fixed in PBS for 10 min. After 4°C. Cells were rinsed and mounted in 10% PBS, 90% glycerol, 1 mg/ml phenylenediamine (mounting medium).

**Peptide re-presentation**

B3Z is a CD8<sup>+</sup> T cell hybridoma that expresses LacZ in response to activation of T cell receptors specific to the SINFEKL peptide (Ova-immunodominant peptide) in the context of H-2K<sup>b</sup> MHC class-I molecules (Karttunen et al., 1992). SINFEKL peptide was crosslinked to gp96 using SPDP (Pierce, Rockford, IL) according to the manufacturer’s protocol. The SINFEKL-conjugated gp96 was dialyzed extensively against PBS, with the final dialysis buffer used to determine background in the assay. Ova-specific peptide presentation was determined by incubating SINFEKL-conjugated gp96 or unconjugated Ova with 10<sup>5</sup> C57BL/6-derived elicited macrophages in the presence or absence of fucoidin, as indicated. Free ligand was then aspirated and the cells washed with medium. The macrophages were then co-cultured with 10<sup>5</sup> B3Z cells for 4 h. Ova re-presentation was assayed by the measurement of LacZ activity using CPRG (Boehringer Mannheim). Alternatively, the K<sup>+</sup>-pecific monoclonal antibody 25-D1.16 Ab (Porgador et al., 1997) was used for FACS analysis of cell surface peptide presentation, as per previously (Berwin et al., 2002a).

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**References**


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