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Cutting Edge: CD91-Independent Cross-Presentation of GRP94(gp96)-Associated Peptides

Brent Berwin,* Justin P. Hart,† Salvatore V. Pizzo, † and Christopher V. Nicchitta 2*†

GRP94(gp96) elicits CD8+ T cell responses against its bound peptides, a process requiring access of its associated peptides into the MHC class I cross-presentation pathway of APCs. Entry into this pathway requires receptor-mediated endocytosis, and CD91 (low-density lipoprotein receptor-related protein) has been reported to be the receptor mediating GRP94 uptake into APC. However, a direct role for CD91 in chaperone-based peptide Ag re-presentation has not been demonstrated. We investigated the contribution of CD91 to GRP94 cell surface binding, internalization, and trafficking in APCs. Whereas a clear role for CD91 in α2-macroglobulin binding and uptake was readily obtained, the addition of excess CD91 ligand, activated α2-macroglobulin, or receptor-associated protein, an antagonist of all known CD91 ligands, did not affect GRP94 cell surface binding, receptor-mediated endocytosis, or peptide re-presentation. These data identify a CD91-independent, GRP94 internalization pathway that functions in peptide Ag re-presentation. *The Journal of Immunology, 2002, 168: 4282–4286.

The molecular chaperone GRP94(gp96) is one of several molecular chaperones that have been shown to elicit anti-tumor responses in murine models (1–5). Recent studies demonstrate that GRP94 functions in both prophylactic and therapeutic protocols to reduce or eliminate tumor growth and progression. The basis for the immunological capabilities of these proteins is thought to reflect two intrinsic properties: 1) they act as general adjuvants to the innate immune system, promoting the maturation and activation of dendritic cells and macrophages and eliciting cytokine secretion (2, 6, 7); and 2) as vehicles for associated peptides, they deliver their bound Ags to professional APCs, to yield peptide-specific T cell stimulation (8–11). This latter function is thought to reflect high-affinity, cell surface chaperone receptors on APCs, which direct GRP94 into the MHC class I re-presentation pathway. It is exclusively the receptor-mediated pathway that is reported to function in the re-presentation of GRP94-associated peptides (8).

Recently, a member of the low-density lipoprotein (LDL) family of scavenger receptors, CD91 (α2-macroglobulin (αM) receptor; LDL receptor-related protein), was proposed to serve as the unique receptor responsible for directing GRP94 into the class-I Ag processing pathways of APCs (12–14). The conclusion that CD91 functions as the GRP94 receptor stems from the observation that αM, the active form (αM*), of which is an established, endogenous ligand for CD91, abrogates GRP94-mediated, APC-dependent T cell stimulation (12). Although consistent with a role for CD91 in GRP94-based Ag re-presentation, previous observations call into question whether the effects of CD91-directed ligands on GRP94-based peptide re-presentation reflect a direct role of CD91 in chaperone uptake and processing. Primarily, CD91 is expressed on a diverse array of cell types, including fibroblasts and hepatocytes, the majority of which do not function as professional APCs (15). Additionally, in affinity chromatography and chemical cross-linking studies of GRP94-interacting proteins, GRP94 was recovered with a single proteolytic product of CD91; no interactions of GRP94 with intact CD91 were reported (12).

In the present study, CD91 function in the receptor-mediated endocytosis and trafficking of GRP94 in APCs was analyzed. It is well established that ligand binding functions of CD91 are regulated by receptor-associated protein (RAP) (16–18), which efficiently blocks the cell surface binding and uptake of all known CD91 ligands (16–19). We report in this work that the binding of GRP94 to APC cell surface receptors was RAP and αM*-insensitive. Furthermore, CD91 and its ligand, *Pseudomonas* exotoxin, segregated from receptor-internalized GRP94 in early compartments of the endocytic pathway. Additionally, re-presentation of GRP94-associated peptides was αM* insensitive. These data identify a primary, CD91-independent re-presentation pathway for GRP94-associated peptides in APCs.

Materials and Methods

Cells and tissue culture

C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA) were used to prepare thioglycollate-elicited peritoneal macrophages. Macrophages were harvested 4–5 days postinjection and enriched by adherence selection.

Abbreviations used in this paper: LDL, low-density lipoprotein; RAP, receptor-associated protein; αM, α2-macroglobulin; CHO, Chinese hamster ovary.

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**Protein purification and labeling**

GRP94 was purified by the method of Wearsch and Nicchitta (20). *Pseudomonas* exotoxin was obtained from Sigma-Aldrich (St. Louis, MO), Texas Red-, biotin-, and fluorescein-succinimidyl esters (Molecular Probes, Eugene, OR) were used to label proteins according to manufacturer’s protocols. Abs against K⁺-OVA complex (25-D1.16) and CD91 were the kind gifts of Dr. J. Yewdell (National Institutes of Health, Bethesda, MD) and Dr. S. Argraves (University of South Carolina, Charleston, SC), respectively. α₂M was purified as previously described (21). Purified α₂M was converted to the CD91-binding, thiol ester-cleaved derivative (H9251 kindly provided pGEX-RAP expression vectors.

**Cell surface binding and uptake**

Receptor-mediated uptake reactions were performed as described previously (23). For confocal microscopy analysis, cells were fixed in 4% paraformaldehyde and mounted under 10% PBS, 90% glycerol, and 1 mg/ml phenylendiamine.

Biotin-GRP94 cell surface receptor binding interactions were analyzed by incubating 10⁶ cells/assay with increasing concentrations of biotin-GRP94 for 30 min on ice. Cells were then washed and resuspended in SDS-PAGE sample buffer, and extracts were prepared for SDS-PAGE. Following transfer to nitrocellulose membranes, biotin-GRP94 levels were determined by ECL, following avidin-HRP detection of biotin-GRP94. Quantification of cell surface-bound biotin-GRP94 was determined against a standard curve prepared from serial dilutions of biotin-GRP94.

Adherent macrophages were incubated at 4°C in RPMI 1640, 0.5% BSA for 30 min with the following ligands: fluorescein-GRP94 and Alexa-α₂M⁺ in the presence or absence of RAP. For competition assays final concentrations used were 25 nM for GRP94 and α₂M⁺ and 2500 nM for RAP (100-fold molar excess). After the 30-min binding period, macrophages were washed and then fixed in 4% paraformaldehyde/PBS. Following fixation, the cells were rinsed and unreacted paraformaldehyde was quenched with 0.05 M NH₄Cl. Coverslips were then mounted onto slides in mounting medium. All images were obtained on a Zeiss LSM 410 laser scanning confocal microscope (Thornwood, NY) using Zeiss LSM version 3.95 software. All image size and contrast adjustments were performed with Photoshop (version 4) software (Adobe Software, Palo Alto, CA).

For study of CD91 binding and RAP competition by FACS, adherent macrophages were incubated for 30 min at 4°C with fluorescein-GRP94 or α₂M⁺-AF488, in the presence or absence of RAP. For competition assays final concentrations were as indicated above. Following incubation, cells were rinsed and fixed in 1% paraformaldehyde. Cells were analyzed for fluorescence by FACS and analysis was performed using CellQuest (BD Biosciences, San Jose, CA).

**Chaperone-based re-presentation**

Peptide re-presentation assays were performed with GRP94 complexed with Sf-InFELK peptide (by heat shock, 15 min at 50°C) (24, 25) and subsequently isolated from free peptide by Sephadex G-75 size exclusion chromatography (Sigma-Aldrich, St. Louis, MO). This preparation does not contain free peptide (26). To assay GRP94/peptide re-presentation, GRP94/peptide complexes and, where indicated, α₂M⁺ were incubated with elicited primary peritoneal macrophages and, following a 3-h incubation at 37°C, subsequently stained with 25-D1.16 Ab (27). The cells were then fixed in 2% paraformaldehyde for FACS analysis.

**Results and Discussion**

**Trafficking itineraries of GRP94 and CD91**

CD91 has been identified as the unique receptor responsible for directing GRP94 into MHC class I re-presentation pathways of APCs (12–14). CD91 is a member of the LDL family of lipoprotein receptors, which bind and internalize an array of ligands, the majority of which are targeted to lysosomes (28). Because little is known regarding the CD91-dependent trafficking of GRP94, the trafficking itineraries of CD91, GRP94, and known CD91 ligands were examined.

To evaluate the subcellular trafficking of CD91 ligands, the trafficking pattern of GRP94 was compared with that of *Pseudomonas* exotoxin, an obligate CD91 ligand (29). In these experiments, fluor-labeled GRP94 and *Pseudomonas* exotoxin were bound to macrophage cell surface receptors, the cells were washed, and the staining pattern was analyzed following warming to 37°C. As is evident in Fig. 1A, receptor-internalized GRP94 (red channel) and *Pseudomonas* exotoxin (green channel) were trafficked to distinct subcellular compartments. The lack of costaining suggested that GRP94 and CD91 were rapidly segregated upon internalization to yield distinct trafficking itineraries. To examine this hypothesis, we determined whether, at early time points, internalized GRP94 and CD91 colocalized. Surprisingly, and as shown in Fig. 1B, GRP94 (red channel) taken up by receptor-mediated endocytosis did not colocalize with internalized CD91 (green channel). This is in direct contrast to the colocalization observed of receptor-internalized GRP94 and IgG (Fig. 1C), which traffic to a FcR⁺ rab5⁺ endosomal compartment (26). In interpreting these data with respect to a role for CD91 in chaperone-mediated cross-presentation, it is important to note that a functional role for CD91 in this process has been proposed not on the basis of direct trafficking studies but rather from the observed inhibition of peptide re-presentation by CD91-specific Abs and by α₂M competition (12). To reconcile these differences, a direct analysis of CD91 function in GRP94 cell surface binding and uptake was performed.

**Analysis of LDL receptor-related protein family activity in receptor-mediated uptake of GRP94**

CD91 is expressed on a diverse array of cell types, including, but not limited to, APCs (15). Previously, we reported that HepG2, a CD91-positive human hepatoma cell line, did not display GRP94 cell surface binding (23, 30). To examine whether there was a positive correlation between CD91 expression and cell surface binding of GRP94, these two parameters were evaluated in an additional CD91-positive cell line, Chinese hamster ovary (CHO), and in RAW264.7 macrophages, a cell line that is CD91 positive and that binds GRP94 (12, 23, 31, 32). As depicted in Fig. 2A, both CHO and RAW264.7 cells express nearly identical levels of CD91, findings consistent with previous reports (14, 31, 32). However, though both cell types were CD91 positive, only RAW264.7 cells display appreciable cell surface binding of GRP94; thus, as previously concluded with regard to HepG2 cells, a positive correlation between CD91 expression and GRP94 cell surface binding could not be demonstrated.

Subsequently, the capacity of the CD91 ligand, α₂M⁺, to compete for GRP94 cell surface binding was examined. Previously, Binder et al. (12) reported that α₂M efficiently inhibits re-presentation of GRP94-associated peptides, as assayed using peptide-dependent stimulation of T cells as a surrogate for receptor function in GRP94 peptide uptake. This observation is surprising, as CD91 ligands generally do not cross-compete for binding (28).

However, a second high-affinity α₂M⁺ binding site has been demonstrated on the surface of APCs (33); thus, it was considered that GRP94 cell surface binding was conferred by this related activity. Therefore, we performed competition experiments with highly purified α₂M⁺, the CD91 binding form of α₂M, or serum, which contains ~2 mg/ml α₂M (34). As shown in Fig. 2B, BSA, BSA supplemented with α₂M⁺, and DMEM plus 10% serum had no effect on the binding of GRP94 to thioglycolate-elicited macrophages. Indeed, GRP94 was observed to bind to and be internalized by elicited macrophages in the presence of 200 μg/ml α₂M⁺ (Fig. 2C). High-affinity binding of GRP94 to macrophage cell surfaces has previously been shown to be specific (8, 13) and saturable (Fig. 2B). Because α₂M⁺ constitutes ~1% of total α₂M (35),
RAP is a 39-kDa protein that acts as a universal antagonist to all known CD91 ligands, including αM* (16, 17, 19). If CD91 functions in the internalization of GRP94, and as αM* has been reported to block the interaction of GRP94 with CD91, RAP would be predicted to antagonize the binding of GRP94 to APCs. Therefore, we tested the effects of RAP on cell surface binding of GRP94, using an established ligand, αM*, as a positive control. Fig. 3B indicates that RAP, at a 100-fold molar excess, did not inhibit GRP94 binding to elicited macrophages. Importantly, under identical assay conditions, αM* binding was efficiently blocked by RAP (Fig. 3C). The inhibition of GRP94 binding by RAP was not due to GRP94 displaying a higher affinity for APCs than αM*, as GRP94 exhibits an overall Kₐ of ∼2 × 10⁻⁷ M to elicited macrophages (Fig. 2B, inset), as compared with a low nanomolar Kₐ for αM* binding to CD91 (36). The effects of RAP on cell surface binding of fluorescein-labeled GRP94 and Texas Red-labeled αM* was also examined by confocal microscopy (Fig. 3, D and E). Consistent with the FACS data, RAP was without effect on GRP94 binding (green channel), whereas αM* binding (red channel) was efficiently blocked (Fig. 3, compare D, minus RAP, with E, plus RAP). Finally, we tested whether αM* directly blocked the re-presentation of GRP94-associated peptides. In these experiments, it was observed that SIINFEKL peptide, complexed with GRP94, was re-presented both in the presence of αM*-containing serum and in the presence of serum supplemented with 100 μg/ml αM* (Fig. 3F), a level previously reported to abolish GRP94-dependent cross-priming of T cells (12).

In noting the marked time interval differences in assays of peptide re-presentation on cell surface class I molecules (≤3 h) and GRP94-mediated T cell activation (>20 h) (12), it should be considered that the reported inhibition of T cell activation by CD91 Ab and αM* may reflect physiological responses of the cells to

Re-presentation of GRP94-associated peptides via a CD91-independent pathway

the αM* levels (200 μg/ml) used in these experiments are equivalent to a total αM* concentration of 20 mg/ml, a concentration in vast excess to that necessary for competition at a shared site(s).

FIGURE 1. Divergent trafficking pathways for GRP94, CD91, and Pseudomonas exotoxin. Texas Red (TR)-GRP94 and fluorescein (Fl)-labeled Pseudomonas exotoxin (PE) (A), fluorescein-labeled Abs against CD91 (B), or fluorescein-labeled IgG (C) were bound to primary peritoneal macrophage cell surface receptors on ice, the cells were washed, and receptor-mediated uptake was initiated by warming the cells to 37°C. After a 30-min (A) or 7-min (B and C) trafficking period, cells were processed for confocal microscopy. Colocalization is observed in the two-channel merge as yellow.

FIGURE 2. CD91-independent cell surface binding of GRP94 to elicited macrophages. A. CHO and RAW264.7 macrophages were incubated with fluorescein-GRP94 on ice, the unbound GRP94 was removed, and the cells were subsequently decorated with anti-CD91 mAb (rhodamine channel). Bound GRP94 and CD91 levels were determined by FACS. B. Fluorescein-GRP94 binding to the cell surface of RAW264.7 macrophages was conducted in the presence of BSA, BSA plus αM* (400 μg/ml), or DMEM plus 10% bovine serum, followed by FACS analysis. Inset, Scatchard analysis of GRP94 binding to macrophages. GRP94 exhibits a Kᵦ of ∼2 × 10⁻⁷ M to macrophages, occupying ∼10⁶ high-affinity sites per cell. C. The effects of the CD91 ligand αM* on the receptor-mediated internalization of GRP94 was examined in RAW264.7 macrophages. Fluorescein-GRP94 was allowed to bind to RAW264.7 macrophages in the presence of 200 μg/ml αM*, the free ligand was removed, and the cells were subsequently warmed to 37°C for 7 min to promote internalization.
prolonged culture with these reagents. For example, ligand-bound CD91 elicits pronounced cell activation; thus, we suggest that CD91 Ab and αM may disrupt the ability of APC to activate cognate T cells (37–39).

In summary, the included data indicate that macrophage CD91 displays biochemical properties consistent with its role as a αM* receptor. However, the lack of a positive correlation between CD91 expression and GRP94 cell surface binding, the absence of colocalization between GRP94 and CD91 in the early trafficking itinerary of the two proteins, the inability of αM* to inhibit the binding or internalization of GRP94 or the re-presentation of its associated peptides, and the observation that RAP, a biological antagonist for CD91 receptor ligands, is without effect on GRP94 binding argue strongly against a role for CD91 in the receptor-mediated internalization of GRP94. From these data, it is equally evident that APCs bear cell surface receptors that are capable of directing GRP94 into the class I Ag re-presentation pathway.

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References


