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Re-Examination of CD91 Function in GRP94 (Glycoprotein 96) Surface Binding, Uptake, and Peptide Cross-Presentation

Angela R. Jockheck-Clark,* Edith V. Bowers,† Mariam B. Totonchy,* Julie Neubauer,‡ Salvatore V. Pizzo,† and Christopher V. Nicchitta* 

GRP94 (gp96)-peptide complexes can be internalized by APCs and their associated peptides cross-presented to yield activation of CD8+ T cells. Investigations into the identity (or identities) of GRP94 surface receptors have yielded conflicting results, particularly with respect to CD91 (LRP1), which has been proposed to be essential for GRP94 recognition and uptake. To assess CD91 function in GRP94 surface binding and endocytosis, these parameters were examined in mouse embryonic fibroblast (MEF) cell lines whose expression of CD91 was either reduced via RNA interference or eliminated by genetic disruption of the CD91 locus. Reduction or loss of CD91 expression abrogated the binding and uptake of receptor-associated protein, an established CD91 ligand. Surface binding and uptake of an N-terminal domain of GRP94 (GRP94.NTD) was unaffected. GRP94.NTD surface binding was markedly suppressed after treatment of MEF cell lines with heparin, sodium chlorate, or heparinase II, demonstrating that heparin sulfate proteoglycans can function in GRP94.NTD surface binding. The role of CD91 in the cross-presentation of GRP94-associated peptides was examined in the DC2.4 dendritic cell line. In DC2.4 cells, which express CD91, GRP94.NTD-peptide cross-presentation was insensitive to the CD91 ligands receptor-associated protein or activated α2-macroglobulin and occurred primarily via a fluid-phase, rather than receptor-mediated, uptake pathway. These data clarify conflicting data on CD91 function in GRP94 surface binding, endocytosis, and peptide cross-presentation and identify a role for heparin sulfate proteoglycans in GRP94 surface binding. The Journal of Immunology, 2010, 185: 6819–6830.

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Abbreviations used in this paper: GRP94.NTD, N-terminal domain of GRP94; Hsp, heat shock protein; HSPG, heparan sulfate proteoglycan; K0, knockout; LDL, low-density lipoprotein; LRP1, low-density lipoprotein receptor-related protein 1; αM, native α2-macroglobulin; αM*, activated α2-macroglobulin; MEf, mouse embryonic fibroblast; pAPC, professional APC; PEA-13, Pseudomonas exotoxin A-resistant MEf; PI, propidium iodide; RAP, receptor-associated protein; siRNA, small interfering RNA; UT, untransfected.

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these findings and identified a broad role for CD91 in the cross-presentation of peptides associated with numerous Hsps and molecular chaperones, including Hsp90, Hsp70, and calreticulin (29).

Given the diversity of tissues that express CD91, it is not immediately apparent how CD91-mediated GRP94–peptide uptake would be restricted to pAPCs, as would be expected if CD91 was the primary, immunologically relevant receptor for Hsps and molecular chaperones. Indeed, after the discovery of CD91 as an Hsp/chaperone receptor, a variety of other surface receptors were identified to function in the recognition and endocytic uptake of Hsps, these findings have engendered controversy regarding the identity of CD91 as an endocytic receptor for GRP94 (31, 35, 36).

To assess directly CD91 function in GRP94 surface binding and internalization, these parameters were examined in both CD91 small interfering RNA (siRNA) knockdown and CD91 knockout (KO) fibroblast cell lines. Whereas the reduction or loss of CD91 expression resulted in markedly decreased RAP binding and internalization, surface binding and endocytic uptake of a recombinantly expressed N-terminal domain of GRP94 (GRP94.NTD) was unaffected. CD91 function in cross-presentation was examined in DC2.4 murine dendritic cells, and it was found that molar excess concentrations of RAP or α2M resulted in either a modest (RAP) or no (α2M) decrease in the cross-presentation of GRP94.NTD-associated peptides. These studies demonstrate that in DC2.4 cells, cross-presentation of GRP94.NTD-associated peptides occurs primarily via fluid-phase, rather than receptor-mediated, uptake and clarify existing data on CD91 function in the cross-presentation of GRP94-associated peptides.

Materials and Methods

Cell culture and siRNA transfection

Mouse embryonic fibroblast 1 (MEF-1) and Pseudomonas exotoxin A-resistant MEF (PEA-13) cells were maintained in DMEM, glucose, l-glutamine, and sodium pyruvate (Cellgro, Manassas, VA), supplemented with 10% FBS (Life Technologies, Durham, NC). DC2.4 cells were maintained in RPMI 1640 with l-glutamine (Cellgro) and cultured according to American Type Culture Collection recommendations. MEF-1 and PEA-13 cells were transfected with 200 nM CD91-directed siRNAs (Qiagen, Valencia, CA) using Lipofectamine2000 (Invitrogen, Carlsbad, CA) and Opti-MEM serum-free media (Life Technologies), according to the manufacturer’s protocol. Cells were maintained in DMEM/10% FBS after transfection and examined for CD91 knockdown efficacy at 24, 48, and 72 h posttransfection by RT-PCR. The siRNA sequences used were LRPI-1 (5'-CAC GTT GAT TAT GCA CAT GAA-3') and LRPI-2 (5'-CTG CCG GTG GTA CAA ATG TAA-3') and were directed against murine LRPI (NM_008512).

Mice

OVA-specific TCR transgenic mice (C57BL/6-Tg[TcrTcr]+100Mjb/I) (OT-1) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the Duke University Animal Facility (Durham, NC), an Association for Assessment and Accreditation of Laboratory Animal Care International-approved facility. All experiments were conducted under an institutional animal care and use committee-approved protocol.

Protein purification

Recombinant canine GRP94.LREK (residues 69–337) was expressed in Escherichia coli strain BL21 as an N-terminal GST fusion (pGEX-2T vector) and purified as previously described (37). After elution from Sepharose 4B glutathione agarose resin (GE Healthcare, Piscataway, NJ), the GST-tag was cleaved using thrombin (Haemotologic Technologies, Essex Junction, VT). Thrombin cleavage was arrested by addition of PMSF, and the protein pool was rechromatographed on a Sepharose 4B glutathione–agarose column. Peak fractions were pooled, loaded onto a HiTrap QHP column (GE Healthcare), and washed with 150 column volumes of cold, sterile depyrogenation buffer (1% Triton X-114, 20 mM Tris, pH 8). Detergent was removed by washing the column with sterile 150 mM NaCl, 20 mM HEPES, pH 7.9, until the A280 returned to baseline. Recombinant protein was then eluted with sterile 750 mM NaCl, 20 mM HEPES, pH 8.0. Peak fractions were pooled and concentrated in a YM-10 centrifugal concentrator (Millipore, Billerica, MA). GRP94.NTD endotoxin content was determined to be ∼15 EU/mg by the QCL-1000 chromogenic limulus amoebocyte lysate assay (Lonza, Walkersville, MD).

Recombinant canine GRP94.NTD (residues 22–337) was expressed in E. coli strain BL21 as a His-tagged fusion (pET15b vector; Novagen, Madison, WI), and purified as described earlier for RAP. After depyrogenation, detergent was removed by washing the column with sterile PBS. Recombinant protein was then eluted with sterile 150 mM imidazole in PBS, pH 7.4. Peak fractions were pooled, concentrated in a YM-10 centrifugal concentrator (Millipore), and dialyzed against sterile PBS.

Recombinant rat RAP was expressed in E. coli strain BL21 as a His-tagged fusion (pLE1 modified pET24a vector) (38). Cultures were induced with 0.1 mM isopropyl-β-D-galactopyranoside for 3 h and bacteria recovered by centrifugation. Bacterial pellets were resuspended in cold buffer A (50 mM dextrose, 50 mM Tris, pH 8, 10 mM imidazole, 300 mM NaCl), and homogenates prepared by French press. Homogenates were incubated with an equal volume of buffer B (10 mM Tris, pH 8, 50 mM KCl, 0.5% [v/v] Tween 20, 0.5% [v/v] Triton X-100, 300 mM NaCl, and 10 mM imidazole) for 30 min on ice. Insoluble debris was subsequently removed by centrifugation at 10,000 × g for 30 min at 4˚C. The supernatant fraction was applied to nickel–Sepharose resin (GE Healthcare) and washed with 30 column volumes of wash buffer (PBS with 0.2% [v/v] Tween 20, 0.2% [v/v] Triton X-100, and 40 mM imidazole, pH 8), and washed overnight in sterile depyrogenation buffer (1% Triton X-114 in PBS, pH 7.5). Recombinant protein was eluted using sterile 150 mM imidazole in PBS, pH 7.9, and dialyzed against sterile PBS. To remove excess detergent, protein was incubated with sterilized, depyrogenated Bio-Beads (Bio-Rad, Hercules, CA) at 1 h at 4˚C and then dialyzed against PBS.

Purification of α2M was performed using endotoxin-free plasma, columns, and buffers and followed a protocol modified from that described previously (39). Mouse plasma was purchased from Harlan (Indianapolis, IN), α2M was separated from mouse plasma by consecutive precipitation with 4% and 16% polyethylene glycol (Fluka, St. Louis, MO), followed by DEAE Sephacel fractionation (Sigma-Aldrich, St. Louis, MO) and purified further over a Sephacryl S-300 sizing column (GE Life Sciences, East Piscataway, NJ). α2M was converted to the activated form (α2M*) by incubation in 200 mM ammonium bicarbonate at 37°C overnight. Activated protein was then dialyzed for 4 h into PBS and stored at 4˚C. Purified protein contained <10 pg endotoxin/mg protein, as determined by Limulus amebocyte lysate assay (Cambrex, Bio Science, Walkersville, MD).

Protein labeling

Proteins were conjugated to Alexa Fluor carboxylic acid succinimidyl esters (Molecular Probes, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, purified protein samples were diluted with sterile 0.15 M sodium bicarbonate, pH 9, and incubated with Alexa Fluor dye (dissolved in sterile DMSO) for 60 min at room temperature in the dark. Free dye was removed using a depyrogenated, PBS-equilibrated Sephadex G-25 column. Proteins were concentrated in a YM-10 or YM-30 centrifugal concentrator (Millipore).

Analytical ultracentrifugation

The m.w. and aggregation state of GRP94.NTD preparations were determined by sedimentation equilibrium ultracentrifugation in a Beckman XL-A analytical ultracentrifuge (Beckman-Coulter, Brea, CA). GRP94.NTD, at concentrations of 0.9, 1.7, and 2.3 mg/ml in 50 mM Tris, pH 8, 100 mM NaCl, 1 mM DTT was analyzed at 10,000 rpm. The partial specific volumes (0.7278) and solvent densities (1.015) were calculated from the amino acid and solvent compositions using the SEDENTERP program.

RT-PCR

Total RNA was extracted from tissue culture cells using TRizol reagent (Invitrogen) according to manufacturer’s specifications and dissolved in nuclelease-free water (Ambion, Austin, TX). RNA concentrations were determined spectrophotometrically, and RNA integrity was assessed using
a 1% formaldehyde gel. Samples were treated with RNase-free DNase I (Ambion) at 37°C for 30 min prior to heat inactivation. cDNA was synthesized from 7.2 μg DNase-treated RNA using Superscript (Invitrogen), according to the manufacturer’s protocol. cDNA was recovered by phenol/chloroform/isooamy alcohol (25:24:1) extraction and sodium acetate/ethanol precipitation and resuspended in 10 mM Tris-Cl, pH 8.5.

PCR was performed using Taq polymerase (Qiagen). The following primer sequences were used: CD91 sense, 5′-ATC ACC CTT CCC GGC AGC TTG-3′; antisense, 5′-AAC CAG GGC CAT CAG GGT CTT TGG-3′; 18S sense, 5′-TCA AGA AGG AAA GTC GGA G-3′; 18S antisense, 5′-GGA CAT CTA AGG GCA TCA CA-3′ (IDT). Thermocycling was conducted in a PTC-200 Thermal Cycler (MJ Research, Waltham, MA) using the following parameters: denaturation at 94°C for 3 min; 33 cycles of 94°C for 30 s, 62°C for 1 min, 72°C for 1 min; final extension at 72°C for 15 min. PCR products were separated on a 1.5% agarose gel and visualized using ethidium bromide. Quantification of band intensities was measured using Image J version 1.38x (National Institutes of Health, Bethesda, MD).

Surface binding and internalization assays

MEF-1 and PEA-13 cultures were rinsed and incubated with prewarmed PBS** (PBS with 1% BSA [Life Technologies], supplemented with 0.22-μm filter-sterilized 0.9 mM CaCl₂ and 0.5 mM MgCl₂) for 30 min at 37°C. Cells were then rinsed with room-temperature PBS without calcium or magnesium (Life Technologies), lifted, and stained as described earlier. To examine the effects of proteolytic cleavage on fluorescence intensity, fluor-conjugated GRP94 was incubated in cytosol buffer until a stable baseline was acquired. Trypsin was then added to the solution and the fluorescence determined at 30-s intervals. Experiments were conducted on a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu Scientific Instruments, Columbia, MD) using an excitation wavelength of 647 nm and an emission wavelength of 665 nm. Relative GFP fluorescence was normalized to the maximum signal acquired during each analysis.

Cross-presentation of GRP94–ova20 complexes

GRP94.NTD–peptide complexes were prepared using ova20 peptide (SGLEQLESINFKELTWS) (NeoBioScience, Cambridge, MA) as previously described (18). Free peptide was removed by dialysis against sterile PBS at 4°C. To assay cross-presentation, DC2.4 cells (2.5 × 10⁶) were cultured with or without 10 μg/ml GRP94.NTD–ova20 complexes at concentrations representing an ~15-fold (αMβ2, 500 μg/ml RAP, or 500 μg/ml RAP, or 500 μg/ml RAP) molar excess of competitor. GRP94.NTD used in competition experiments was prepared in the absence of ova20 peptide, as previously described (18). Cells were incubated for 4 h under standard conditions, rinsed with PBS containing calcium and magnesium to remove unbound protein, and replenished with 100 μl complete media. OT-1 splenocytes were harvested from MHC class I-restricted OVA-transgenic mice, and single-cell suspensions were prepared by repeated subcapsular injection of complete culture media (high glucose phenol red-free DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, and 50 μM 2-ME). The DC2.4 cells were then cocultured with 2.5 × 10⁶ OT-1 splenocytes. Cell supernatants were harvested after 24 h and stored at −20°C. IFN-γ levels were quantified using DUO-ELISA kits (R&D Systems, Minneapolis, MN).

Statistics

Values reported in all analyses are expressed as the mean ± SD. Differences between groups were analyzed using a paired Student t test. Statistical significance was accepted at p < 0.01.
GRP94.NTD (residues 22–337) and GRP94.LREK (residues 69–337) were produced by recombinant expression, purified, and depyrogenated. Both forms were purified to near homogeneity and migrated predominately as monomers in native PAGE (Fig. 1A, 1B). After incubation at 42°C for 30 min, both GRP94.NTD and GRP94.LREK formed higher-order oligomers that displayed reduced mobility in native PAGE, as described in previous publications (42). Analytical ultracentrifugation analyses confirmed that GRP94.LREK behaved as a primarily homogeneous species of 41 kDa at 4°C (Fig. 1C). Because GRP94.NTD and GRP94.LREK behaved comparably in all assays, the term GRP94.NTD will be used interchangeably to describe both constructs.

**Cell surface binding of GRP94.NTD is independent of CD91 expression**

CD91 was the first receptor proposed to function in GRP94 surface binding and endocytosis (26). The initial experimental evidence supporting a physiological function of CD91 in GRP94 surface binding and uptake was primarily indirect and derived from the observations that GRP94-mediated peptide cross-presentation was inhibited by CD91 ligands (26). More recently, siRNA-mediated knock-down of CD91 expression was shown to markedly suppress cell surface GRP94 binding and GRP94-mediated peptide cross-presentation in RAW264.7 macrophage cells (30). Although these data are consistent with an essential role for CD91 in GRP94 surface recognition and endocytic uptake, the observed inhibition of cross-presentation was only seen with a GRP94-ova20 complex; no inhibition was seen with a GRP94–SIINFEKL complex (26).

To examine CD91 function in GRP94 surface recognition, binding studies were conducted in CD91-expressing MEF-1 cells that had been transfected with either of two CD91-directed siRNAs (LRP1_1 or LRP1_2). The effects of LRP1_1 and LRP1_2 on CD91 expression were monitored by RT-PCR. At 24 h post-transfection, cells transfected with LRP1_2 displayed a 75% reduction in CD91 mRNA levels compared with that of untransfected and mock transfection controls (Fig. 2A, 2B). Transfection with LRP1_1 had no effect on CD91 mRNA levels and was used as a control for off-target effects. Plots of relative PCR product intensity versus PCR cycle number indicated that amplification of the CD91 target sequence in the mock and LRP1_1-transfected cells was within the linear range (Fig. 2A). Neither siRNA affected GRP94.NTD surface binding to MEF-1 cells. MEF-1 cells were transiently transfected with either of two CD91-targeting siRNAs (LRP1_1 and LRP1_2) or a vector only (mock) control and examined for changes in CD91 expression 24 h posttransfection. A, Total RNA samples were isolated, and CD91 mRNA knockdown efficiency was determined by RT-PCR. CD91 cDNA from mock-transfected cells displayed linear amplification between PCR cycles 25 and 33 ($R^2 = 0.9974$). B, Untransfected (UT), mock-transfected, and siRNA-transfected cells were examined for changes in CD91 mRNA levels using RT-PCR. All samples were tested for DNA contamination using a paired transcriptase-deficient (RT ) reaction and were examined for off-target effects to 18S rRNA. C and D, MEF-1 cells were transfected with LRP1_2 siRNA or a mock control and analyzed by flow cytometry. CD91 surface expression was determined using 10 μg/ml RAP (C), and GRP94.NTD surface binding was conducted with 25 μg/ml GRP94.NTD (D): unstained cells (thin lines), stained cells (bold lines), mock-transfected cells (gray lines), and LRP1_2 siRNA transfected cells (black lines). Mock-transfected and untransfected cells displayed identical RAP and GRP94.NTD binding (data not shown). The data presented are representative of three independent replicates.

CD91 surface expression was assayed by monitoring the surface binding of fluorescently labeled RAP using flow cytometry. RAP, a 39-kDa endoplasmic reticulum-resident chaperone, binds multiple sites on the ectodomain of CD91 and is known to inhibit binding of all known CD91 ligands (43). Compared with mock-transfected cells, RAP binding to LRP1_2-transfected cells was reduced by 45%, a finding consistent with prior results (Fig. 2C) (26). These data demonstrate that CD91 expression was significantly reduced after transfection with LRP1_2, yet mock- and LRP1_2-transfected cells bound equivalent levels of fluorescently labeled GRP94.NTD (Fig. 2D). Transfection with LRP1_1 did not affect RAP or GRP94.NTD binding and served as a negative control (data not shown). These data suggest that GRP94.NTD binds to receptor sites other than CD91.

Although RAP is an antagonist of all established CD91 ligands, it is also known to bind to other LDL family receptors, and thus RAP competition studies are not uniquely diagnostic of CD91 function (43). Therefore, additional experimental approaches were used to examine CD91-dependent versus CD91-independent GRP94.NTD surface interactions. To this end, RAP and GRP94.
The interaction of RAP and GRP94.NTD with cell surface binding sites expressed by MEF-1 and PEA-13 cells was further characterized in ligand titration studies. In these experiments, RAP displayed high-affinity, saturable binding to MEF-1 cells, with near-maximal inhibition occurring at 10 μg/ml RAP (Fig. 4A). Conversely, PEA-13 cells displayed very low RAP binding activity over the same concentration range. Discriminable RAP binding to PEA-13 cells was only observed at concentrations that markedly exceeded those necessary to saturate RAP binding to MEF-1 cells. To examine this low-affinity binding phenomenon in greater detail, RAP binding titrations were performed with both cell lines (Fig. 4B). Both MEF-1 and PEA-13 cells displayed linear, low-affinity/high-capacity RAP binding, with no saturation evident at RAP concentrations up to 100 μg/ml. In contrast, GRP94.NTD binding to MEF-1 and PEA-13 cells was linear at concentrations up to 25 μg/ml (Fig. 4C, 4D) and essentially linear up to 100 μg/ml. As previously observed, PEA-13 cells bound more GRP94.NTD than did MEF-1 cells at all concentrations examined. These low-affinity/high-capacity binding interactions indicate that GRP94.NTD interacts with surface sites that are highly expressed by both MEF-1 and PEA-13 cells.

Cell surface HSPGs are binding sites for GRP94.NTD

The GRP94.NTD binding characteristics reported above are similar to the low-affinity/high-capacity binding interactions observed for numerous cell surface HSPG ligands. HSPGs are also known to bind a diverse array of protein ligands and serve as coreceptors for numerous surface receptors, including CD91 (45–53). Additionally, GRP94 is known to bind heparin, which has a structure similar to HSPG polysaccharide side chains (54–56).

To determine if cell surface HSPGs contribute to GRP94.NTD surface binding to MEF-1 and PEA-13 fibroblasts, three experimental approaches were used. First, MEF-1 and PEA-13 cells were incubated with increasing concentrations of heparin, washed, and then analyzed by flow cytometry. Results are expressed as the mean of three independent experiments ± SD.

While CD91 expression was undetectable in PEA-13 cells, it was characterized in ligand titration studies. In these experiments, RAP displayed high-affinity, saturable binding to MEF-1 cells, with half-maximal and maximal binding observed at ~0.2 and 1.0 μg/ml, respectively (Fig. 4A). Conversely, PEA-13 cells displayed very low RAP binding activity over the same concentration range. Discriminable RAP binding to PEA-13 cells was only observed at concentrations that markedly exceeded those necessary to saturate RAP binding to MEF-1 cells. To examine this low-affinity binding phenomenon in greater detail, RAP binding titrations were performed with both cell lines (Fig. 4B). Both MEF-1 and PEA-13 cells displayed linear, low-affinity/high-capacity RAP binding, with no saturation evident at RAP concentrations up to 100 μg/ml. In contrast, GRP94.NTD binding to MEF-1 and PEA-13 cells was linear at concentrations up to 25 μg/ml (Fig. 4C, 4D) and essentially linear up to 100 μg/ml. As previously observed, PEA-13 cells bound more GRP94.NTD than did MEF-1 cells at all concentrations examined. These low-affinity/high-capacity binding interactions indicate that GRP94.NTD interacts with surface sites that are highly expressed by both MEF-1 and PEA-13 cells.

Identification of low-affinity/high-capacity RAP and GRP94.NTD binding activities

The interaction of RAP and GRP94.NTD with cell surface binding sites expressed by MEF-1 and PEA-13 cells was further characterized in ligand titration studies. In these experiments, RAP displayed high-affinity, saturable binding to MEF-1 cells, with half-maximal and maximal binding observed at ~0.2 and 1.0 μg/ml, respectively (Fig. 4A). Conversely, PEA-13 cells displayed very low RAP binding activity over the same concentration range. Discriminable RAP binding to PEA-13 cells was only observed at concentrations that markedly exceeded those necessary to saturate RAP binding to MEF-1 cells. To examine this low-affinity binding phenomenon in greater detail, RAP binding titrations were performed with both cell lines (Fig. 4B). Both MEF-1 and PEA-13 cells displayed linear, low-affinity/high-capacity RAP binding, with no saturation evident at RAP concentrations up to 100 μg/ml. In contrast, GRP94.NTD binding to MEF-1 and PEA-13 cells was linear at concentrations up to 25 μg/ml (Fig. 4C, 4D) and essentially linear up to 100 μg/ml. As previously observed, PEA-13 cells bound more GRP94.NTD than did MEF-1 cells at all concentrations examined. These low-affinity/high-capacity binding interactions indicate that GRP94.NTD interacts with surface sites that are highly expressed by both MEF-1 and PEA-13 cells.

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To determine if cell surface HSPGs contribute to GRP94.NTD surface binding to MEF-1 and PEA-13 fibroblasts, three experimental approaches were used. First, MEF-1 and PEA-13 cells were incubated with increasing concentrations of heparin, washed, and then analyzed by flow cytometry. Results are expressed as the mean of three independent experiments ± SD.
It is well established that cell surface HSPGs can facilitate ligand binding to CD91 (50, 52). As expected, these conditions significantly inhibited both of HSPG glycan chains during their biosynthesis (57–62). After culturing cells in sodium chlorate-supplemented media for 24 h, GRP94.NTD and RAP binding was examined by flow cytometry. This treatment resulted in a nearly complete loss of GRP94.NTD binding activity to both MEF-1 and PEA-13 cells but had no significant effect on RAP binding (Fig. 5B). After sodium chlorate treatment, a substantial (>90%) reduction in HSPG sulfation was confirmed using the monoclonal anti-heparan-sulfate Ab 10E4 (Fig. 5C).

Lastly, MEF-1 and PEA-13 cells were treated with heparinase II, which cleaves sulfated glycan chains, and then assayed for GRP94.NTD and RAP surface binding. Digestion with heparinase II resulted in a substantial reduction in GRP94.NTD binding at all concentrations examined (Fig. 5D). It did not, however, alter RAP binding (Fig. 5E). Digestion of PEA-13 cells with heparinase II also resulted in a significant reduction in GRP94.NTD surface binding (data not shown). A substantial (>90%) decrease in cell surface HSPG sulfation was confirmed using the anti-heparan-sulfate Ab 10E4 (Fig. 5F). These data demonstrate that disruption of HSPG surface structures significantly reduces GRP94.NTD but not RAP binding to MEF-1 and PEA-13 cells and identify a role for HSPGs in GRP94.NTD cell surface binding. At present, we do not know the identity (or identities) of the HSPGs involved in GRP94.NTD surface binding.

**RAP and GRP94.NTD are internalized via pathways that are kinetically and spatially distinct**

It is well established that cell surface HSPGs can facilitate ligand binding to CD91 (50, 52). This is believed to occur through either the formation of ligand–HSPG complexes that are recognized by CD91 or through the “transfer” of HSPG-bound ligands to CD91 for subsequent internalization and/or processing (50, 52). These findings are of particular relevance to the conflicting data regarding the role of CD91 in GRP94 uptake, as they suggest that CD91 could function in GRP94 endocytosis without directly contributing to GRP94 recognition at the cell surface.

To determine if CD91 supports GRP94.NTD internalization, the kinetics of both RAP and GRP94.NTD internalization were examined. MEF-1 cells were incubated with either RAP or GRP94.NTD on ice, washed, and then warmed to 37°C to allow internalization of surface-bound proteins. At the indicated intervals, cells were rapidly cooled and subjected to surface proteolysis to remove residual surface-bound ligands. As would be predicted of a typical CD91 ligand, RAP was rapidly endocytosed by MEF-1 cells, with 80–90% of the total surface-bound RAP becoming resistant to external proteases within 5 min (Fig. 6A). The kinetics of GRP94.NTD internalization was considerably slower than that determined for RAP, with little uptake being observed before 20 min. Because CD91 internalization rates are known to be unusually high, the relatively sluggish rate of GRP94.NTD internalization argues against a significant role for CD91 in GRP94.NTD endocytosis (28, 63). In further support of CD91-independent modes of internalization for GRP94.NTD, MEF-1 and PEA-13 cells internalized GRP94.NTD at nearly identical rates (data not shown).

To determine if CD91 directly functions in GRP94.NTD internalization, GRP94.NTD uptake experiments were conducted in the presence of excess RAP. In these experiments, cells were preincubated with saturating levels of RAP on ice prior to GRP94.NTD surface binding. Because CD91 is rapidly internalized and recycled back to the cell surface, a large molar excess of unlabeled RAP was included in the internalization buffer to ensure that recycling CD91 molecules would be complexed by extracellular RAP. As expected, these conditions significantly inhibited both
Because the uptake assay used in these experiments measures both internalization and intracellular processing, these data could be interpreted to indicate that RAP and GRP94.NTD differ significantly in their relative sensitivity to postinternalization processing. The observed time-dependent increase in cell-associated fluorescence would then reflect the kinetics of intracellular processing, rather than the kinetics of ligand uptake. To address this alternative interpretation, the kinetics of RAP and GRP94.NTD endocytosis were visualized by confocal microscopy. MEF-1 cells were incubated with RAP and GRP94.NTD on ice, washed, warmed to 37°C to allow internalization, rapidly cooled at the indicated intervals, fixed, and examined by confocal microscopy (Fig. 6C–F). In agreement with the kinetic analyses of RAP and GRP94.NTD uptake measured by flow cytometry, RAP was fully internalized within 15 min (Fig. 6D). Conversely, GRP94.NTD remained at the cell periphery and was internalized via a substantially slower endocytic route. These micrographs also revealed that RAP and GRP94.NTD seldom colocalize either at the cell surface or during endocytosis. Together, these data demonstrate that RAP and GRP94.NTD are internalized via distinct, independent pathways.

GRP94.NTD that had been internalized by cells for 60 min exhibited a level of fluorescence that exceeded the initial signal from surface-associated protein (Fig. 6B). It has previously been reported that proteolysis of fluor-conjugated proteins can result in the loss of intramolecular quenching and a subsequent increase in fluorescence yield (64, 65). To determine if fluor-conjugated GRP94.NTD behaved in a similar manner, GRP94.NTD integrity and fluorescence were monitored after digestion with trypsin (Fig. 7A, 7B). Aliquots of the digestion reactions were removed at the indicated time points, and GRP94.NTD integrity was visualized by SDS-PAGE. Fractional loss of full-length GRP94.NTD was then quantified using ImageJ (Fig. 7A, 7B). In parallel studies, GRP94.NTD fluorescence intensity was assayed. As depicted in Fig. 7B, the time-dependent proteolytic degradation of fluor-conjugated GRP94.NTD was associated with an increase in total sample fluorescence. This suggests that the time-dependent increase in fluorescence observed after GRP94.NTD endocytic uptake is due to the proteolytic processing of the internalized protein in an intracellular compartment(s). To test this hypothesis further, MEF-1 cells were treated with the protease inhibitor leupeptin prior to and during GRP94.NTD uptake (Fig. 7C, 7D). This treatment effectively suppressed the previously observed increase in fluorescence, reducing the total GRP94.NTD signal to the same level as the initial surface-bound GRP94.NTD signal. Together, these data demonstrate that fluor-conjugated GRP94.NTD undergoes proteolytic processing in endosomal compartments, a finding consistent with previous studies demonstrating the exchange of GRP94-associated peptides onto mature MHC class I molecules in a post-endoplasmic reticulum compartments (35).

**FIGURE 6.** GRP94.NTD and RAP are internalized via spatially and kinetically distinct pathways. A and B, MEF-1 cells were incubated with either 10 μg/ml RAP or 25 μg/ml GRP94.NTD, washed, warmed to 37°C for 0, 5, 20, or 60 min, and placed on ice to arrest endocytosis. Residual surface-bound ligands were removed by proteolysis on ice. The percentage protease-resistant signal was calculated by normalizing the protease-resistant signal to the total signal at each time point. Results are expressed as the mean of three independent experiments ± SD. B, Comparison of MEF-1 cells treated either as in A (control) or treated with 10 μg/ml RAP prior to GRP94.NTD incubation and excess RAP during internalization (RAP Competition). Residual surface-bound ligands were removed by proteolysis on ice. The dashed gray bar indicates total GRP94.NTD surface binding prior to treatment with extracellular protease. C–F, MEF-1 cells were incubated with 5 μg/ml RAP (red) and 40 μg/ml GRP94.NTD (blue), washed, and warmed to 37°C for 0 (C), 5 (D), 15 (E), or 60 (F) min to allow internalization. Cells were then fixed and processed for confocal microscopy. Original magnification ×100. Scale bars, 20 μm. All data presented are representative of three independent replicates.

**RAP surface binding and internalization (data not shown).** However, these conditions had no effect on either the quantity or the rate of GRP94.NTD endocytosis (Fig. 6B).

The data presented above demonstrate that CD91 does not directly bind GRP94.NTD or contribute significantly to GRP94.NTD internalization by MEF-1 and PEA-13 cells. Although these findings are inconsistent with the observation that CD91 ligands inhibit CD94-mediated peptide cross-presentation by DC2.4 cells is insensitive to exogenous CD91 ligands

**FIGURE 6.** GRP94.NTD and RAP are internalized via spatially and kinetically distinct pathways. A and B, MEF-1 cells were incubated with either 10 μg/ml RAP or 25 μg/ml GRP94.NTD, washed, warmed to 37°C for 0, 5, 20, or 60 min, and placed on ice to arrest endocytosis. Residual surface-bound ligands were removed by proteolysis on ice. The percentage protease-resistant signal was calculated by normalizing the protease-resistant signal to the total signal at each time point. Results are expressed as the mean of three independent experiments ± SD. B, Comparison of MEF-1 cells treated either as in A (control) or treated with 10 μg/ml RAP prior to GRP94.NTD incubation and excess RAP during internalization (RAP Competition). Residual surface-bound ligands were removed by proteolysis on ice. The dashed gray bar indicates total GRP94.NTD surface binding prior to treatment with extracellular protease. C–F, MEF-1 cells were incubated with 5 μg/ml RAP (red) and 40 μg/ml GRP94.NTD (blue), washed, and warmed to 37°C for 0 (C), 5 (D), 15 (E), or 60 (F) min to allow internalization. Cells were then fixed and processed for confocal microscopy. Original magnification ×100. Scale bars, 20 μm. All data presented are representative of three independent replicates.
addition of excess RAP or α2M* did not reduce GRP94.NTD surface binding to DC2.4 cells (Fig. 8C). GRP94.NTD surface binding was also not suppressed by α2M, which is not recognized by CD91. CD91 expression by DC2.4 cells was confirmed by RT-PCR (data not shown).

To determine if CD91 facilitates GRP94.NTD internalization/processing by DC2.4 cells, cells were preincubated with RAP, α2M*, or α2M on ice, followed by incubation with labeled GRP94.NTD on ice. The cells were then rinsed and warmed to 37°C in the absence or presence of excess ligand. After 60 min, the cells were chilled to arrest endocytosis and analyzed by flow cytometry. Neither α2M* nor α2M significantly reduced GRP94.NTD internalization/processing (Fig. 8D). RAP modestly (~15%) decreased the GRP94.NTD signal.

To determine if CD91 ligands indirectly influence GRP94-mediated peptide cross-presentation, cross-presentation studies were conducted using OT-1 splenocytes. OT-1 mice express a transgenic TCR that recognizes the OVA-based OV A257–264 (SIINFEKL) epitope. For these studies, GRP94.NTD was complexed to ova2 (SGLEQLE-SIINFEKLTEWTS) using a previously published protocol (18) and dialyzed to remove uncomplexed ova20 peptide. Because of its length, ova20 must be internalized and processed to yield the SIINFEKL epitope.

DC2.4 cells were pulsed with the GRP94.NTD–ova20 complex in the absence or presence of CD91 ligands for 4 h at 37°C. Cells were then washed, incubated with OT-1 splenocytes, and CTL activation assayed 24 h later by IFN-γ ELISA. DC2.4 cells pulsed with GRP94.NTD–ova20 activated OT-1 splenocytes, and DC2.4 cells pulsed with uncomplexed GRP94.NTD were unable to stimulate OT-1 splenocytes (Fig. 8E). In the absence of CD91, DC2.4 cells pulsed with RAP, α2M*, or α2M did not elicit OT-1 IFN-γ expression (data not shown). In contrast with prior findings, neither RAP, α2M*, nor α2M significantly reduced GRP94-mediated ova20 cross-presentation (Fig. 8F).

As an additional control, excess uncomplexed GRP94.NTD was used to compete GRP94.NTD–ova20 cross-presentation (Fig. 8F).
Because GRP94-mediated peptide cross-presentation has been reported to occur through receptor-mediated endocytosis, it was expected that GRP94-mediated ova20 cross-presentation would be significantly diminished in the presence of 50-fold molar excess of GRP94.NTD. However, GRP94.NTD–ova20 cross-presentation was not significantly reduced under these conditions, suggesting that nonspecific fluid-phase uptake serves as the primary internalization pathway for GRP94.NTD–ova20 in DC2.4 cells.

To distinguish between the receptor-mediated and fluid-phase components of GRP94.NTD internalization in DC2.4 cells, a modified internalization assay was conducted. In these experiments, fluor-conjugated RAP or GRP94.NTD was added directly to the internalization buffer, and uptake studies were performed at 37°C in the continued absence or presence of excess unlabeled ligand. Endocytosis was then arrested by the addition of ice-cold buffer and the samples analyzed by flow cytometry. As depicted in Fig. 8G, a 10-fold molar excess of unlabeled RAP inhibited RAP internalization/processing by 80% during the first 20 min of internalization. A similar inhibition was observed after 5 min of internalization (data not shown). These data distinguish the mechanisms of GRP94.NTD and RAP internalization by DC2.4 cells and are consistent with the RAP and GRP94.NTD binding characteristics depicted in Fig. 8A and 8B, respectively. The inclusion of a 10-fold excess unlabeled RAP inhibited GRP94.NTD internalization by ~20% in DC2.4 cells, a finding that mirrors the RAP-dependent decrease in GRP94.NTD internalization/processing observed in Fig. 8D.

**Discussion**

In this study, we report three primary findings. 1) CD91 is dispensable for GRP94.NTD binding and endocytosis. Comparisons of surface binding and uptake functions of CD91+/+ and CD91−/− mouse embryonic fibroblasts demonstrated that CD91 expression was required for the surface binding and endocytic uptake of the CD91 ligand RAP, but not GRP94.NTD. Additionally, when present in molar excess, the CD91 ligands RAP or α2M displayed little to no competition for either surface binding of GRP94.NTD or cross-presentation of GRP94.NTD–ova20 complexes by DC2.4 cells. 2) In DC2.4 cells, fluid-phase, rather than receptor-mediated, pathways represent the primary mechanism of GRP94.NTD–peptide internalization and entry into the cross-presentation pathway. 3) HSPGs function as GRP94.NTD cell surface binding sites on mouse embryonic fibroblast cell lines. This latter observation extends the diversity of cellular binding interactions that participate in the surface recognition of Hspgs and molecular chaperones and suggests that HSPG-dependent interactions may contribute to the biology of GRP94-elicited immune responses.

HSPGs are ubiquitously expressed, structurally diverse proteoglycans that contain highly heterogeneous, sulfated polysaccharide side chains. Their cell surface expression patterns vary by cell/tissue type and developmental stage and can be altered in response to extracellular stimuli such as tissue injury (59). Additionally, the HSPG structure itself can be modified during its biosynthesis and through interactions with extracellular proteases. These characteristics make HSPGs uniquely poised to play a broad variety of physiological roles in ligand binding, processing, and signal transduction (59). The included data demonstrate that cell surface HSPGs can serve as binding sites for extracellular GRP94.NTD. This conclusion was derived from three distinct experimental approaches. First, disrupting HSPG surface structures with heparin resulted in diminished GRP94.NTD binding to both MEF-1 cells and CD91-deficient PEA-13 cells. Second, modulating HSPG sulfation by culturing cells in sodium chloride-supplemented media resulted in the dramatic loss of GRP94.NTD surface binding activity. Third, altering cell surface HSPG structures by digestion with exogenous heparinase II resulted in decreased GRP94.NTD surface binding by both MEF-1 and PEA-13 cell lines. Thus, by multiple independent methods, HSPGs have been implicated in the direct surface recognition of GRP94.NTD.

These data demonstrate that cell surface HSPGs are necessary for a significant portion of GRP94.NTD surface binding to MEF-1 and PEA-13 cells. With such a prominent contribution to GRP94.NTD surface binding, it is curious that this interaction was not previously reported. As depicted in Figs. 4 and 8, HSPG-dependent GRP94.NTD surface binding is a low-affinity/high-capacity binding process. This is in contrast with the high-affinity interactions observed between GRP4 and the scavenger receptors SR-A and SREC-I (31, 32). Thus, if the cells used in previous studies expressed high-affinity GRP4 receptors such as SR-A and SREC-I, the contributions of low-affinity/high-capacity receptors such as cell surface HSPGs could have gone unnoticed. Alternatively, but not exclusively, the immunological cell lines used in previous studies may not have expressed the specific class(es) of HSPGs that contribute to GRP94.NTD surface binding on MEF-1 and PEA-13 cells.

CD91 was the first endocytic receptor proposed to function in GRP94 recognition and uptake (26). In these studies, Binder et al. (26, 29, 30) demonstrated that GRP94-mediated peptide cross-presentation was almost completely suppressed by α2M, RAP, and an anti-CD91 blocking Ab. In a subsequent study, it was shown that siRNA-mediated knock-down of CD91 expression ablated GRP94-mediated peptide cross-presentation by RAW264.7 cells (30). Together, these studies directly implicate CD91 in the cross-presentation of GRP94-associated peptides and suggest strongly that CD91 functions as a GRP94 endocytic receptor. To examine further the role of CD91 as a GRP94 endocytic receptor, our studies focused on the ability of CD91 to mediate three processes that are required for GRP94-mediated peptide cross-presentation: surface binding, endocytosis, and intracellular processing. We first characterized the contribution of CD91 to GRP94.NTD surface binding by using both siRNA-mediated knockdown and CD91 KO systems. Reduction or loss of CD91 expression did not diminish GRP94.NTD surface binding, and excess GRP94.NTD did not compete for RAP surface binding. These findings are inconsistent with a direct role for CD91 in the surface binding of GRP94.NTD. It is difficult to reconcile these data with prior reports demonstrating that siRNA-mediated loss of CD91 expression blocked GRP94 surface binding and cross-presentation activity (30). However, it is not clear how these investigators obtained a complete silencing of CD91 expression without selecting for the siRNA-transfected RAW264.7 cells; in the absence of selection, siRNA-dependent silencing of CD91 expression would be limited to that fraction of the cell population undergoing productive transfection.

Our findings are in agreement with several past reports concluding that CD91 does not contribute to the surface recognition of GRP94, calreticulin, or Hsp70 (36, 66, 67). In this regard, it should be noted that both Berwin et al. (36) and Binder and Srivastava (30) demonstrated that neither a 100-fold molar excess of RAP nor a 100-fold molar excess of α2M inhibited GRP94 binding to RAW264.7 mouse macrophage cells. However, Binder and Srivastava also demonstrated that a partial (~60%) competition be-
between these ligands could be achieved if cells were chemically fixed prior to ligand binding. A later study demonstrated that a 50-fold molar excess of α2M reduced GRP94 surface binding by 35% to fixed primary human PBMCs (68). Because chemical fixation was required to observe the binding competition, the physiological relevance of these results remains unclear.

Although CD91 does not directly bind GRP94.NTD at the cell surface, CD91 could influence GRP94.NTD endocytosis through alternative mechanisms. In fact, it is well established that CD91 can facilitate ligand internalization and metabolism without significantly contributing to ligand recognition at the cell surface (48, 50, 52). The ability of CD91 to mediate GRP94 internalization was thus examined by studying the uptake kinetics of RAP and GRP94.NTD by MEF-1 cells. Our results demonstrated that GRP94.NTD and RAP were internalized via kinetically and spatially distinct pathways, indicating that these ligands have divergent mechanisms of uptake and intracellular sorting. These observations are in agreement with past studies by Berwin et al. (36) who demonstrated that the CD91 ligand Pseudomonas exotoxin A did not colocalize after 30 min of internalization by RAW264.7 cells. Additionally, GRP94.NTD uptake kinetics were nearly identical in MEF-1 and PEA-13 cells (data not shown), and the continuous presence of RAP during internalization had no significant effect on either the quantity or rate of GRP94.NTD endocytosis by MEF-1 cells. Together, these data demonstrate that CD91 does not directly contribute to GRP94.NTD endocytosis by MEF-1 cells. It remains to be determined if CD91 forms coreceptor complexes that are unique to RAW264.7 cells and in this way contributes to GRP94 surface binding and endocytosis.

Finally, we examined if CD91 ligands indirectly influenced the cellular processing of GRP94.NTD. Our experiments demonstrate that α2M* did not significantly reduce GRP94.NTD surface binding, intracellular processing, or GRP94.NTD-mediated ova20 cross-presentation by DC2.4 cells. Whereas RAP had a modest (~15%) effect on GRP94.NTD processing, it had no effect on GRP94.NTD–ova20 cross-presentation, a finding that contrasts with previous reports demonstrating that a 50-fold molar excess of RAP suppressed GRP94–ova20 cross presentation by ~70% (30). Our observations are, however, in agreement with Berwin et al. (36) who demonstrated that α2M* did not inhibit the cross-presentation of GRP94–SIINFEKL complexes by RAW264.7 cells. Conversely, our data contradict reports that identify CD91 as having an essential role in GRP94-mediated peptide cross-presentation (26, 30). Notably, these past studies used a commercial source of α2M that was reported to contain a mixture of activated and α2M, though such preparations are primarily composed of α2M, which is not a ligand for CD91 (30). Because α2M is a broad-spectrum protease inhibitor, we also determined if α2M could affect GRP94-mediated peptide cross-presentation independently of CD91, perhaps via suppression of intracellular proteolytic processing events. We conclude that in DC2.4 cells, α2M does not inhibit GRP94.NTD surface binding, internalization/processing, or GRP94.NTD-mediated ova20 cross-presentation.

It is important to note that GRP94.NTD-mediated ova20 cross-presentation was not inhibited in the presence of 50-fold excess GRP94.NTD. Combined with the experiments that measured the continuous internalization of GRP94.NTD, these studies demonstrate that DC2.4 cells can internalize and process GRP94.NTD–ova20 through a fluid-phase pathway. Although it is well established that GRP94-mediated peptide cross-presentation can occur through receptor-mediated endocytosis, the contribution of fluid-phase uptake to GRP94-mediated peptide cross-presentation has not been widely studied. Several studies have established that pAPCs can internalize extracellular GRP94 though fluid-phase endocytosis (4, 35). Additionally, it is well established that exogenous Ags can access the cross-presentation pathway via fluid-phase uptake (69–72). In light of these findings, it is unclear how CD91 ligands could efficiently ablate GRP94-mediated peptide cross-presentation in RAW264.7 cells (30). RAW264.7 cells are known to internalize exogenous ligands through fluid-phase uptake and would be expected to be competent for cross-presentation of GRP94–peptide complexes internalized via this pathway (73–75). Indeed, in the assay conditions used by these investigators, cells are cultured in the presence of GRP94 and CD91 ligands for 20 h, and thus uptake via fluid-phase pathways would be expected to contribute a significant fraction of the internalized GRP94 (26, 29, 30).

Combined, our data clarify the existing controversies regarding CD91 function in GRP94 surface binding and endocytic uptake and establish fluid-phase uptake as a prominent mechanism for internalization of GRP94–peptide complexes into the cross-presentation pathways of APCs. In addition, these studies established HSPGs as cell surface GRP94 binding sites. The challenge now confronting the field is to understand the precise molecular mechanism(s) of GRP94-mediated peptide cross-presentation, as well as its physiological relevance.

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Disclosures
The authors have no financial conflicts of interest.

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dendritic cells interact with gp96 via CD91 and regulate inflammatory responses. 


