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The Cytoplasmic Domain of the Low Density Lipoprotein (LDL) Receptor-related Protein, but Not That of the LDL Receptor, Triggers Phagocytosis*

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The macrophage LDL receptor and LDL receptor-related protein (LRP, CD91) mediate the phagocytic-like uptake of atherogenic lipoproteins and apoptotic cells, yet the structural basis of their phagocytic functions is not known. To address this issue, we transfected macrophages with chimeric proteins containing the cytoplasmic tails and transmembrane regions of the LDL receptor or LRP and the ectodomain of CD2, which can bind non-opsonized sheep red blood cells (SRBCs). Macrophages expressing receptors containing the LDL receptor domains were able to bind but not internalize SRBCs. In contrast, macrophages expressing receptors containing the cytoplasmic tail of LRP were able to bind and internalize SRBCs. Chimeras in which the LRP cytoplasmic tail was mutated in two di-leucine motifs and a tyrosine in an NP*X*Y*XXX*L motif were able to endocytose anti-CD2 antibody and bind SRBCs, but SRBC phagocytosis was decreased by 70%. Thus, the phagocytic-like functions of LRP, but not those of the LDL receptor, can be explained by the ability of the LRP cytoplasmic tail to trigger phagocytosis. These findings have important implications for atherogenesis and apoptotic cell clearance and for a fundamental cell biological understanding of how the LDL receptor and LRP function in internalization processes.

The ability of macrophages to internalize large particles by the process known as phagocytosis represents a key property of this cell type (1). A variety of macrophage receptors have been demonstrated to mediate phagocytosis, including receptors that bind to the IgG-Fc domain, mannose residues, and complement after these molecules have opsonized cells. The process of phagocytosis involves a cascade of signaling reactions that orchestrate changes in the actin cytoskeleton and the delivery of internal membranes to the engulfing regions of the macrophage plasma membrane. These signaling reactions are coordinated by the recruitment of actin- and membrane-related signal transduction molecules, including tyrosine kinases, by the cytoplasmic tails of these phagocytic receptors (1). Indeed, the cytoplasmic domains of the receptors mentioned above can trigger phagocytosis in chimeric receptors containing ectodomains from non-phagocytic receptors (2–5).

Certain members of the low density lipoprotein (LDL)[1] receptor family have been implicated in important phagocytic-like processes and have therefore been referred to as "phagocytic receptors." LDL receptor-related protein (LRP, CD91) has been reported to participate in the phagocytosis of apoptotic cells by macrophages (6–8). In addition, LRP has been shown to play a role in the uptake of matrix-retained and aggregated LDL by macrophages and aggregated LDL by smooth muscle cells (9, 10). Moreover, work from a number of laboratories has shown that the LDL receptor itself mediates the uptake of large LDL aggregates (9, 11–15). The internalization of matrix-retained LDL by LRP and aggregated LDL by the LDL receptor are thought to contribute to the critical event of foam cell formation during atherogenesis (16). Both of these processes can be distinguished from receptor-mediated endocytosis by their increased susceptibility to inhibitors of actin polymerization, actin signaling molecules (*e.g.* Rac1 and Cdc42), myosin, phosphatidylinositol 3-kinase, and tyrosine kinases (9, 11–15). Despite these findings and the physiological importance of LRP- and LDL receptor-mediated phagocytic-like processes, there has been no direct proof that either receptor can directly mediate phagocytosis.

With this background, the goal of the current study was to apply a rigorous cell biological test to the question of whether LRP and the LDL receptor can function like known phagocytic receptors, such as IgG-Fc receptors and the mannose receptor. As alluded to above, the test is based upon the idea that phagocytic receptors contain amino acid sequences in their cytoplasmic tail, or tail plus transmembrane region, that are able to direct the uptake of large particles (*e.g.* red blood cells (RBCs)) using their own or heterologous ectodomains (2–5). Our data show that whereas the LDL receptor fails this critical test, the cytoplasmic tail of LRP can, in fact, direct RBC phagocytosis in chimeric receptor model. These findings have important implications for mechanisms of foam cell formation and apoptotic cell clearance and for a fundamental cell biological understanding of how these two important members of the LDL receptor family function in internalization processes.

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[1] The abbreviations used are: LDL, low density lipoprotein; BDM, 2,3-butanedione monoxime; HDL, high density lipoprotein; LPDS, lipoprotein-depleted serum; LRP, low density receptor-related protein; SMase, sphingomyelinase; SRBC, sheep red blood cell.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media were purchased from Fisher Scientific; L-glutamine, penicillin/streptomycin, and phosphate-buffered saline (PBS) were from Invitrogen, and fetal bovine serum was from Gemini Bio-Products, Inc. Tissue culture flasks and plates were from Corning Glass Works (Corning, NY). LDL was isolated from human plasma by preparative ultracentrifugation at $d = 1.063$ g/dl (17) and was labeled with ^{125}I using Iodogen-coated tubes (Pierce) and Na[^{125}I] as described previously (18); the labeled LDL had a specific activity of 150–300 cpm/ng protein and was used within 3 weeks of iodination. Lipoprotein-depleted serum (LPDS) was prepared from fetal bovine serum by preparative ultracentrifugation at $d = 1.21$ g/dl. Radioactive iodine (Na[^{125}I]; 17.4 Ci/mg) was purchased from PerkinElmer Life Sciences. Blocking and non-blocking antibodies against LRP were made as previously described (19). Preimmune and immune rabbit anti-LDL receptor rabbit IgG was kindly provided by Drs. Loren Fong and Allen Cooper (Stanford University Medical School and Palo Alto Medical Foundation, Palo Alto, CA) *Bacillus cereus* sphingomyelinase (SMase), cytochalasin D, 2,3-butanedione monoxime (BDM), bovine serum albumin (BSA, essentially fatty acid-free), EDTA tetrasodium salt, and Hepes were products of Sigma Chemical. Genistein and LY 294002 (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one) were from Biomol (Plymouth Meeting, PA). CD2 DNA and MC1061 cells were a gift from Dr. Brian Seed (Massachusetts General Hospital, Boston, MA). Superfect transfection reagent was from Qiagen (Valencia, CA). Sheep red blood cells (SRBCs) were purchased from Colorado Serum Company (Denver, CO). The following antibodies were purchased: mouse anti-human CD2 antibody (Serotec, Inc., Raleigh, NC); phycoerythrin-labeled CD2 antibody (BD PharMingen, San Diego, CA); Alexa-594 goat anti-mouse IgG (Molecular Probes, Eugene, OR); rabbit antiserum to SRBCs (Cappel, ICN Biomedicals Inc., Irvine, CA); and Cy-2-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Organic solvents as well as all other reagents were from Fisher Scientific.

Cell Culture—RAW-LR5 macrophages were derived from RAW 264.7 cells as described (20) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cell types were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

^{125}I-LDL Degradation Experiments—Aggregated ^{125}I-LDL was prepared by vortexing 250 μg/ml ^{125}I-LDL at the highest setting for 30 s. RAW-LR5 macrophages were plated at ~50% confluency and incubated the next day with 20 μg/ml vortex-aggregated ^{125}I-LDL for 24 h. The media were assayed for ^{125}I-LDL degradation as described (9). In some experiments, the macrophages were incubated with the lipoproteins for 5 h and then chased in medium without labeled LDL for an additional 19 h.

Chimeric Receptor Construction—First, a CD2 ectodomain construct flanked by *Hin*dIII and *Bam*H1 sites was prepared by PCR from CD2 cDNA (see Fig. 2). Next, the following five constructs were prepared by RT-PCR from RNA isolated from RAW-LR5 macrophages: (*a*) cytoplasmic tail of the LDL receptor flanked by *Mlu*I and *Not*I, (*b*) cytoplasmic tail plus transmembrane domain of the LDL receptor flanked by *Bam*H1 and *Not*I, (*c*) cytoplasmic tail of LRP flanked by *Mlu*I and *Not*I, (*d*) cytoplasmic tail plus transmembrane domain of LRP flanked by *Bam*HI and *Not*I, and (*e*) cytoplasmic tail plus transmembrane domains of γ-chain (γ:γ) flanked by *Bam*H1 and *Not*I. In addition, a separate construct containing the cytoplasmic tail plus transmembrane domain of LRP was subjected to site-directed mutagenesis to effect alanine substitutions for both di-leucine sequences and for the tyrosine in the NPVY sequence, as indicated in Fig. 2. Using PCR, a construct containing the transmembrane domain and the mutated tail flanked by *Bam*H1 and *Not*I was made. To create CD2:CD7:LDLR, we began with the construct with CD16:CD7:lck in pCDM8 (4). CD16 was replaced with CD2 using the *Hin*dIII and *Bam*HI sites to obtain CD2:CD7:lck, and lck was then replaced with the LDLR cytoplasmic tail construct using the *Mlu*I and *Not*I sites. To construct CD2:LDLR:LDLR, the CD7:lck was replaced with LDLR:LDLR using the *Bam*HI and *Not*I sites. The same strategy was used to create the three LRP containing constructs and CD2:γ:γ, using the fragments listed above.

Transient Transfection of RAW-LR5 Macrophages—RAW-LR5 macrophages were transiently transfected with the constructs described above in pCDM8 using Superfect transfection reagent following the manufacturer's protocol. Briefly, on day 1, the macrophages were plated in wells in 12-well dishes so that they were ~70% confluent on day 2. At that time, the cells were transfected using 2.5 μg of DNA and 5 μl of Superfect per well for 3–4 h. The transfection mix was then removed, and cells from each well were replated onto three round coverslips in wells of 24-well dishes. The transfected macrophages were assayed for CD2 expression, antibody endocytosis, and SRBC binding and internalization on day 3.

Assays for CD2 Expression, Endocytosis, and SRBC Binding and Internalization—After washing five times in BWD buffer (125 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 5 mM dextrose, 10 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM Hepes, pH 7.4), 2×10^6 SRBCs in 300 μl were added to each well of transfected macrophages and incubated at 37 °C for 25 min. To assay cell surface CD2 expression, the cells were incubated on ice with 1:400 mouse anti-CD2 antibody for 30 min, followed by 1:400 Alexa-594 goat anti-mouse IgG. The cells were then fixed with 3.7% formaldehyde and viewed by fluorescence microscopy using an Olympus IX 70 inverted microscope equipped with an RS-Photometrics Cool Snap CCD camera (Roper Scientific). Images were acquired using Roper Scientific RS Image software. To assay endocytosis by the chimeric receptors, which also served as the assay for CD2 expression in macrophages transfected with the LRP-containing constructs, the cells were incubated with 1:20 phycoerythrin (PE)-labeled anti-CD2 antibody for 30 min at 37 °C, and then fixed with 3.7% formaldehyde and viewed using an LSM 510 laser scanning confocal microscope (Zeiss) equipped with Zeiss LSM Image 5 Image Browser software. To assay SRBC binding and internalization, the macrophages were fixed with 2% glutaraldehyde and viewed by phase microscopy. In certain experiments, simultaneous staining for cellular CD2 and SRBCs was done as follows: after incubation with SRBCs, the macrophages were fixed with 3.7% formaldehyde and then incubated for 5 min with BWD buffer containing 0.2% Triton X-100. The cells were then rinsed and incubated with both 1:20 PE-labeled anti-CD2 antibody and 1:400 rabbit antiserum to SRBCs, followed by incubation with 1:400 Cy-2-labeled goat anti rabbit IgG. To assay phagocytosis, non-internalized SRBCs were removed by hypotonic lysis using 0.2% NaCl. The macrophages were then fixed in 2% glutaraldehyde and viewed by phase microscopy using the Olympus IX 70 inverted microscope described above. Phagocytic index was assessed by averaging the number of internalized SRBCs in macrophages with at least one internalized SRBC.

Statistics—All bar graph values are given as means ± S.E. ($n = 3$); missing error bars in the bar graphs signify S.E. values smaller than the graphic symbols.

RESULTS

The LDL Receptor Mediates the Uptake and Degradation of Vortex-aggregated ^{125}I-LDL by RAW-LR5 Macrophages via a Phagocytic-like Process—To explore the phagocytic-like properties of the LDL receptor in a readily transfectable macrophage (20), we studied the ability of RAW-LR5 macrophages to internalize and degrade large, vortexed-induced aggregates of ^{125}I-LDL. LDL aggregates are abundant in atherosclerotic lesions and likely contribute to macrophage foam cell formation during atherogenesis (16). As shown in Fig. 1*A*, these macrophages internalize and degrade vortex-aggregated ^{125}I-LDL. Most importantly, aggregated ^{125}I-LDL was up-regulated by sterol depletion and markedly suppressed by sterol loading to a degree almost identical to that seen with the standard LDL receptor ligand, monomeric LDL (Fig. 1*A* and *inset*). Therefore, RAW-LR5 macrophages, like other macrophages (11, 12, 21, 22), internalize and degrade large aggregates of LDL by a mechanism that is strongly dependent on the LDL receptor.

We next sought to determine if the degradation of aggregated ^{125}I-LDL by RAW-LR5 macrophages was susceptible to inhibitors of actin polymerization and other processes that are often used to distinguish phagocytosis from receptor-mediated endocytosis. We utilized four such inhibitors: cytochalasin D (actin); LY294002 (phosphatidylinositol 3-kinase); BDM (myosin); and genistein (tyrosine kinases) (9). As shown in Fig. 1*B*, each of the four inhibitors decreased the degradation of aggregated ^{125}I-LDL to a substantially greater degree than that of monomeric ^{125}I-LDL, which is internalized by receptor-mediated endocytosis. Thus, RAW-LR5 macrophages internalize and degrade aggregated LDL by a process that is largely dependent on the LDL receptor and on a variety of cellular processes that are often associated with phagocytosis.

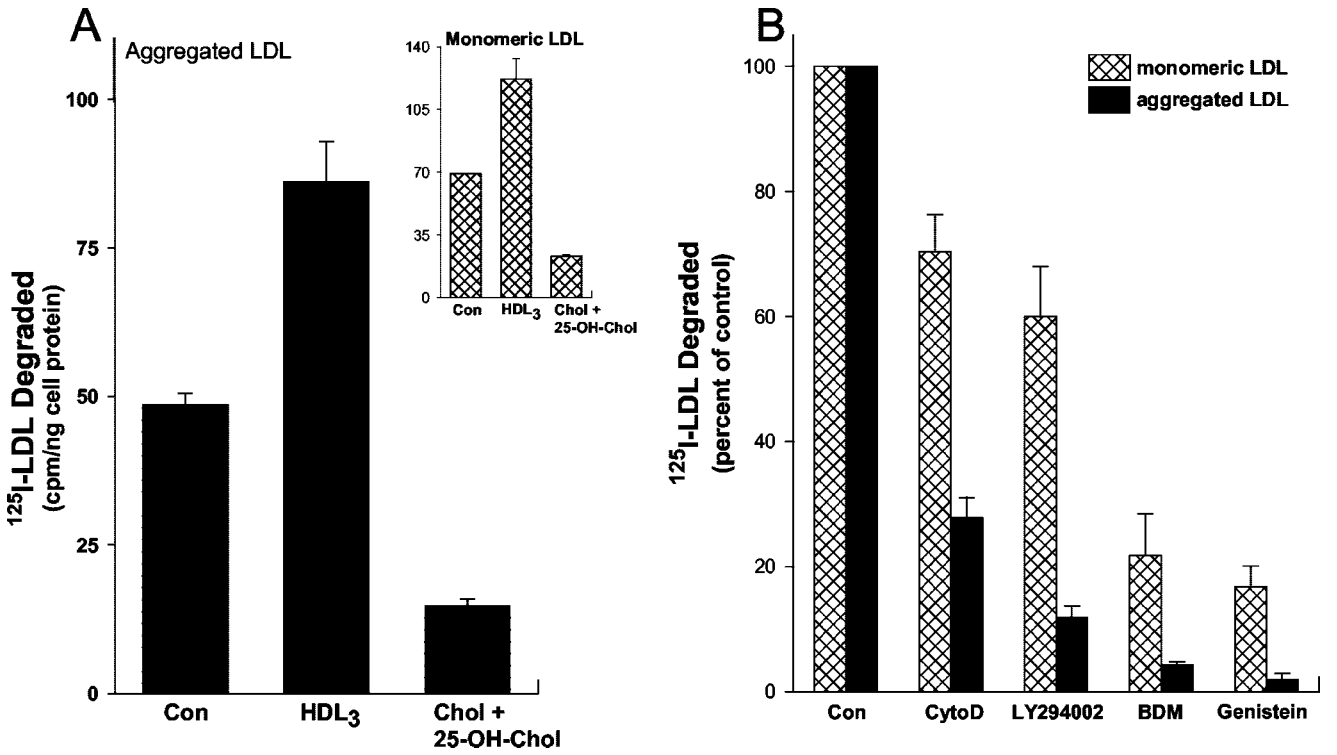

FIG. 1. **The LDL receptor mediates the uptake and degradation of vortex-aggregated 125I-LDL by RAW-LR5 macrophages via a phagocytic-like process.** *A*, RAW-LR5 macrophages with no additional treatments (*Con*) or treated as described below were incubated with 20 μg/ml vortexed-aggregated ^{125}I-LDL for 5 h and then assayed for ^{125}I-LDL degradation. *HDL$_3$*, cells were preincubated for 24 h in medium containing 10% lipoprotein-deprived serum (LPDS) and 300 μg/ml HDL$_3$ to up-regulate LDL receptors; *Chol+25OHC*, cells were preincubated for 24 h in 20 μg/ml cholesterol and 2 μg/ml 25-hydroxycholesterol to down-regulate LDL receptors. *Inset*, macrophages preincubated with HDL$_3$ or cholesterol plus 25-hydroxycholesterol were incubated with 20 μg/ml of non-retained, monomeric ^{125}I-LDL for 5 h and then assayed for degradation. *B*, RAW-LR5 macrophages were preincubated for 24 h in medium containing LPDS and HDL$_3$ to up-regulate LDL receptors. The cells were then incubated directly with 20 μg/ml monomeric or vortexed-aggregated ^{125}I-LDL for 5 h (*Con*) or pretreated for 30 min with 1 μM cytochalasin D, 100 μM LY 294002, 170 μM genistein, or 25 mM BDM and then incubated with the labeled LDL. At the end of the 5-h incubation period, the macrophages were chased in medium without labeled LDL for an additional 19 h before assaying ^{125}I-LDL degradation. The inhibitors were included throughout the entire 24-h incubation period.

The Cytoplasmic Tail and Transmembrane Regions of the LDL Receptor Are Not Sufficient to Mediate the Phagocytosis of Sheep Red Blood Cells—In view of the above data, we sought to determine if the LDL receptor had a key structure-function property of known phagocytic receptors, namely, the ability of its cytoplasmic tail with or without its transmembrane region to direct the internalization of large particles using a chimeric receptor system (2–5). To carry out these experiments, we took advantage of an experimental system described by Allen and co-workers (5). In this system, chimeric receptors containing the ectodomain of the T cell surface protein CD2, which binds the LFA molecule of non-opsonized sheep red blood cells (SRBCs), are expressed in macrophages (5). These CD2-expressing macrophages, unlike control macrophages, can bind these SRBCs. Most importantly, when the chimeric CD2 molecule contains the cytoplasmic tail of a *bona fide* phagocytic receptor, like that of FcγRII, the transfected macrophages can phagocytose non-opsonized SRBCs (5).

In this context, we created two constructs containing the CD2 ectodomain and the LDL receptor cytoplasmic tail (Fig. 2, *first two constructs*). The transmembrane region of the first construct (CD2:CD7:LDLR) contained the CD7 transmembrane domain, which was used previously in similar phagocytosis experiments (4). The transmembrane region of the second construct (CD2:LDLR:LDLR) contained the transmembrane domain of the LDL receptor itself. As a positive control, we also created a CD construct (CD2:γ:γ) in which the transmembrane and cytoplasmic domains were from γ-chain (Fig. 2, *sixth construct*), a molecule that mediates the phagocytosis of FcγRI and

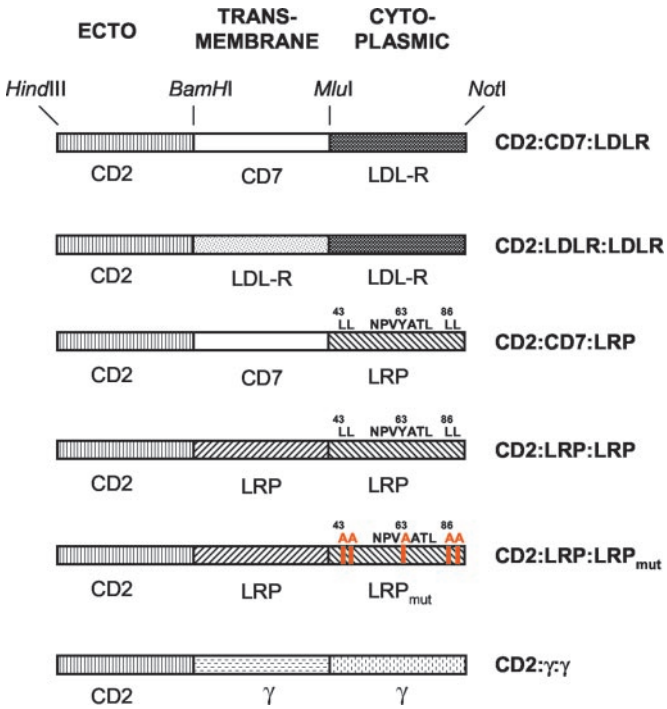

FIG. 2. **CD2-containing chimeric receptor constructs.** The constructs used in this study contained the ectodomain of CD2 and the transmembrane and cytoplasmic domains of either the LDL receptor, LRP, mutated cytoplasmic tail of LRP (LRP$_{mut}$), or γ-chain.

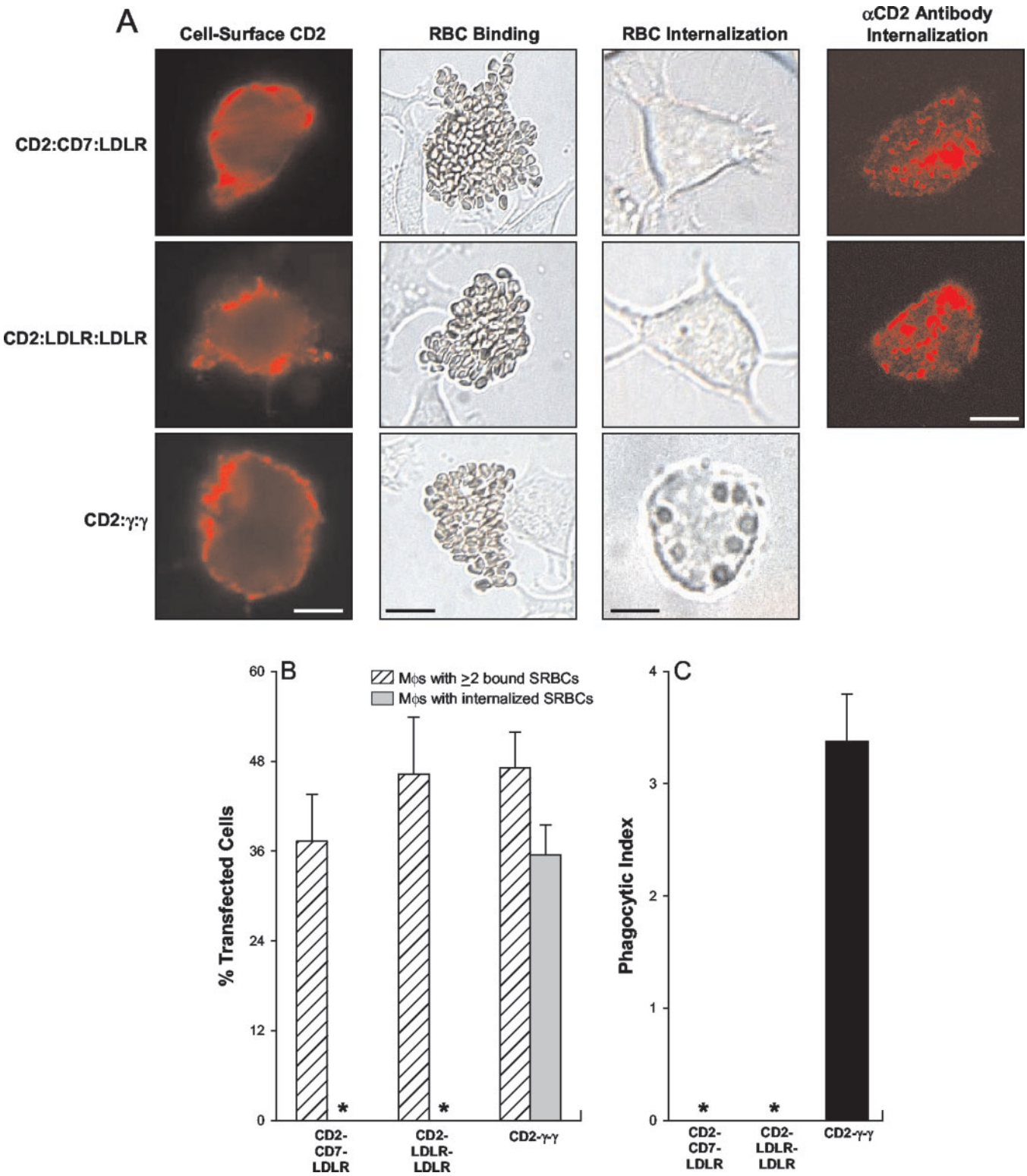

FIG. 3. **The cytoplasmic tail and transmembrane regions of the LDL receptor are not sufficient to mediate the phagocytosis of SRBCs.** *A*, RAW-LR5 macrophages transfected with CD2:CD7:LDLR, CD2:LDLR:LDLR, or CD2L:γ:γ were incubated with SRBCs and then assayed for cell surface expression of CD2, binding and internalization of SRBCs, and endocytosis of anti-CD2 antibody. *Scale bars*, 10 μm. *B* and *C*, Quantification of SRBC binding and internalization in transfected macrophages and phagocytic index based upon the observation of three groups of 1000 macrophages for each condition (see Table I for details). The *asterisks* in *panel C* indicate no internalized SRBCs were observed in 3000 macrophages.

RIII. RAW-LR5 macrophages were transiently transfected with these constructs and assayed for the following: expression of cell surface CD2 via binding of anti-CD2 antibody to fixed cells, followed by Alexa-594-labeled secondary antibody; cell surface binding and internalization (phagocytosis) of SRBCs by phase microscopy before and after hypotonic lysis of the SRBCs, respectively; and internalization of fluorescently labeled monomeric anti-CD2 antibody (endocytosis) via confocal microscopy.

To validate the experimental system, we first showed that CD2-expressing macrophages transfected with CD2:γ:γ were

TABLE I

Transfection efficiency, SRBC binding, and SRBC internalization in macrophages transfected with chimeric receptors containing the CD2 ectodomain and the transmembrane and/or cytosolic tail domains of CD7, γ-chain, the LDL receptor, and LRP

Transfection efficiency was determined by the percentage of macrophages expressing CD2 as assessed by cell-surface anti-CD2 antibody staining in the LDLR- and γ-chain-containing constructs or by internalization of anti-CD2 antibody in the LRP-containing constructs. For SRBC binding and internalization, the data listed under "% of transfected cell" were estimated by adjusting the denominator based on the transfection efficiency data: [(# per 1000 cells) ÷ (transfection efficiency)] × 10. Phagocytic index indicates the average number of internalized SRBCs in macrophages with at least one internalized SRBC.

Construct	Transfection efficiency (% Mϕs expressing CD2)	Macrophages with ≥ 2 bound SRBCs		Macrophages with internalized SRBCs		Phagocytic index (average # SRBCs/Mϕ[a])
		# Mϕs per 1000 cells[a]	% of transfected Mϕ[a]	# Mϕs per 1000 cells[a]	% of transfected Mϕs[a]	
Mock	NA[b]	0.7 ± 0.3	NA[b]	0.3 ± 0.3	NA[b]	0
CD2:CD7:LDLR	5.1 ± 1.0	19.0 ± 3.2	37.3 ± 6.3	0	0	0
CD2:LDR:LDLR	4.1 ± 0.8	19.0 ± 3.1	46.3 ± 7.6	0	0	0
CD2:γ:γ	9.2 ± 1.4	43.3 ± 4.4	47.1 ± 4.8	32.7 ± 3.7	35.5 ± 4.0	3.4 ± 0.4
CD2:CD7:LRP	4.3 ± 0.7	4.3 ± 0.5[c]	10.0 ± 1.1[c]	5.3 ± 1.2	12.3 ± 2.8	1.2 ± 0.1
CD2:LRP:LRP	5.3 ± 0.3	6.8 ± 1.8[c]	12.8 ± 3.4[c]	10.0 ± 2.1	18.9 ± 3.9	1.5 ± 0.1

[a] Means ± S.E. for three sets of fields with 1000 cells/field.
[b] NA, not applicable in mock-transfected cells.
[c] While >80% of the SRBC-binding macrophages transfected with LDLR- and γ-chain-containing chimeric receptors bound > 4 RBCs and displayed a typical rosetting pattern, only 20% of the SRBC-binding macrophages transfected with the LRP-containing chimeric receptors bound > 4 RBCs.

able to both bind and phagocytose SRBCs (Fig. 3*A*, *third row* of images). Internalization of the SRBCs was confirmed by showing that they were not labeled by an anti-SRBC antibody under non-permeabilization conditions but were labeled in permeabilized macrophages (data not shown). In stark contrast, macrophages expressing CD2:CD7:LDLR or CD2:LDLR:LDLR, while showing excellent SRBC binding, were completely unable to internalize the SRBCs (Fig. 3*A*, *first* and *second row* of images). Mock-transfected macrophages were unable to bind or internalize SRBCs (Table I). When the data were quantified for a large number of cells (three groups of cells of 1000 each) and corrected for transfection efficiency, all three chimeric receptors were able to direct SRBC binding to a similar degree (37–47% of transfected cells), but only CD2:γ:γ was able to direct SRBC internalization (35.5% of transfected cells; see Table I and Fig. 3*B*). For those CD2:γ:γ-transfected macrophage internalizing at least one SRBC, the average number of SRBCs per macrophages was 3.4 ± 0.4 (Table I and Fig. 3*C*). To prove the functionality of the two LDL receptor-containing chimeric receptors, we showed that both were able to mediate the endocytosis of a monomeric ligand (Fig. 3, *last column* of images). Thus, neither the LDL receptor cytoplasmic tail nor the combination of the transmembrane region and tail is able to trigger the phagocytosis of bound SRBCs.

The Cytoplasmic Tail of LRP Is Sufficient to Mediate the Phagocytosis of SRBCs—Although LRP does not participate in the internalization of aggregated LDL by macrophages, it does play an important role in the phagocytic-like internalization of extracellular matrix-retained LDL, which is another type of atherogenic LDL in lesions (9). Moreover, LRP has also been reported to participate in the phagocytosis of apoptotic cells by macrophages (6–8). We therefore determined whether the cytoplasmic tail with or without the transmembrane region of this receptor could trigger phagocytosis of SRBCs in the CD2-chimeric receptor system.

Initial observations revealed that macrophages transfected with the two LRP-containing constructs (Fig. 2, *third* and *fourth constructs*) behaved differently from macrophages transfected with LDL receptor- or γ-chain-containing constructs. Consistent with the data of Li *et al.* using LRP minireceptors (23), the steady-state level of CD2 on the cell surface was difficult to detect, but the endocytosis of fluorescently labeled anti-CD2 antibody was robust (Fig. 4*A*, *first column* of images). Although the recycling kinetics of LRP, which is known to be highly enriched in recycling endosomes (24), has not be explored in detail, our initial findings indicate that the cytoplasmic tail of LRP is sufficient to impart this property to a chimeric receptor. Likely related to this observation, we also noted that the number of SRBCs bound per cell was much less than that observed with macrophages transfected with the LDL receptor- or γ-chain-containing constructs (compare *second column* of images in Figs. 4*A* and 3*A*).

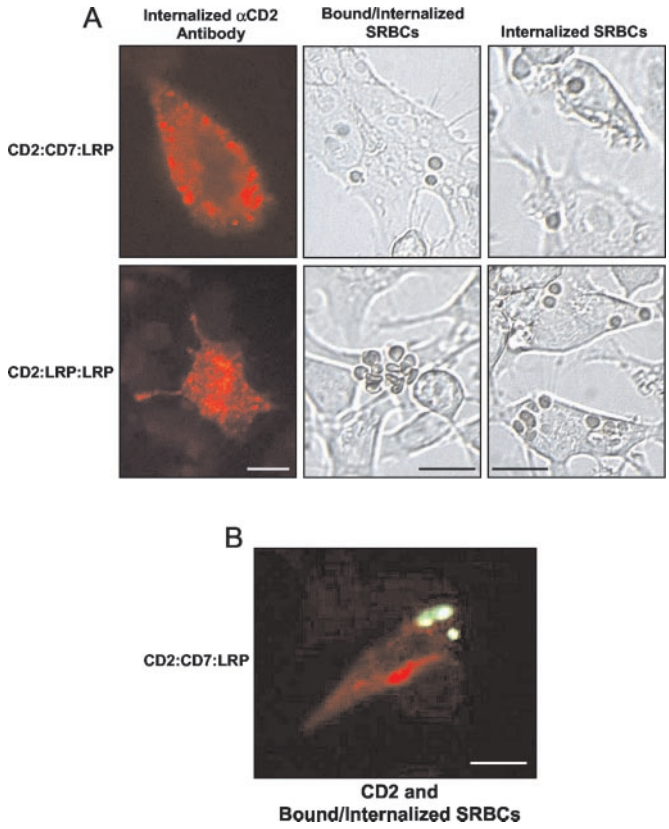

FIG. 4. **The cytoplasmic tail of LRP is sufficient to mediate the phagocytosis of SRBCs.** *A*, RAW-LR5 macrophages transfected with CD2:CD7:LRP or CD2:LRP:LRP were incubated with SRBCs and then assayed for endocytosis of anti-CD2 antibody and binding and internalization of SRBCs. *B*, macrophage transfected with CD2:CD7:LRP was incubated with SRBCs and then stained for both CD2 and SRBCs, showing that the SRBCs were internalized by a transfected macrophage. *Scale bars*, 10 μm.

Despite these differences in cell surface receptor expression and SRBC binding, a relatively high percentage of transfected macrophages was able to internalize at least 1–2 SRBCs per

cell. Representative images are shown in Fig. 4*A*, *third column* of images, and quantitative data based on three sets of 1000 cells for SRBC binding, internalization, and phagocytic index are listed in Table I, and the binding and internalization data are displayed in the first two sets of bars in Fig. 6. While the phagocytic index was not as high as that seen with CD2:γ:γ, which was expressed on the cell surface to a much greater degree than the LRP-containing receptors, it is important to note that there was no SRBC internalization with either mock-transfected macrophages or macrophages transfected with the LDL receptor-containing constructs (Table I). As expected, macrophages immunostained for both CD2 (*red*) and SRBCs (*green*) demonstrated that the internalized SRBCs were found in transfected macrophages (Fig. 4*B*). Thus, chimeric receptors containing the LRP cytoplasmic tail, either with or without the transmembrane region, are able to bind and internalize SRBCs.

Receptor Internalization and Signaling Motifs in the Cytoplasmic Tail of LRP Determine the Steady-state Localization and Phagocytic Capacity of the Chimeric Receptor—The cytoplasmic tail of LRP contains two di-leucine motifs and an NP*XYXXX*L motif that have been shown to affect the cell surface localization of LRP and the rate of endocytosis of LRP ligand in LRP-transfected CHO cells (23). We therefore transfected RAW-LR5 macrophages with a CD2-containing construct containing the CD2 ectodomain, the LRP transmembrane domain, and a mutagenized LRP cytoplasmic tail in which all four leucine residues and the tyrosine residue of the NP*XYXXX*L motif were changed to alanine residues (Fig. 2, *fifth construct*; CD2:LRP:LRP_{mut}). Consistent with the data of Li *et al.* (23), we found that this mutant chimeric receptor was more readily detected on the cell surface than CD2:LRP:LRP (Fig. 5*A*). We also found that macrophages transfected with CD2:LRP:LRP_{mut} bound SRBCs to a greater extent than those transfected with CD2:LRP:LRP (*inset* of Fig. 6). Indeed, some of the transfected cells displayed a rosetting pattern (Fig. 5, *B* and *C*).

We next examined the ability of the mutant chimeric receptor mediate endocytosis and phagocytosis. Although the initial rate of endocytosis might be expected to be decreased (23), macrophages transfected with the mutant receptor were able to readily endocytose anti-CD2 antibody over a period of 30 min (Fig. 5*B*). Remarkably, however, there was relatively little phagocytosis of SRBCs by macrophages transfected with CD2:LRP:LRP_{mut}. Whereas 18.9 ± 3.9% of transfected CD2:LRP:LRP-transfected macrophages internalized SRBCs, this value was only 5.9 ± 0.9% in CD2:LRP:LRP_{mut}-transfected macrophages (~70% decrease) (Fig. 6 and Table I). Thus, one or more of three motifs explored in this experiment, namely, the two di-leucine motifs and the tyrosine residue of the NP*XYXXX*L motif, are necessary to trigger full phagocytic activity in the chimeric receptor.

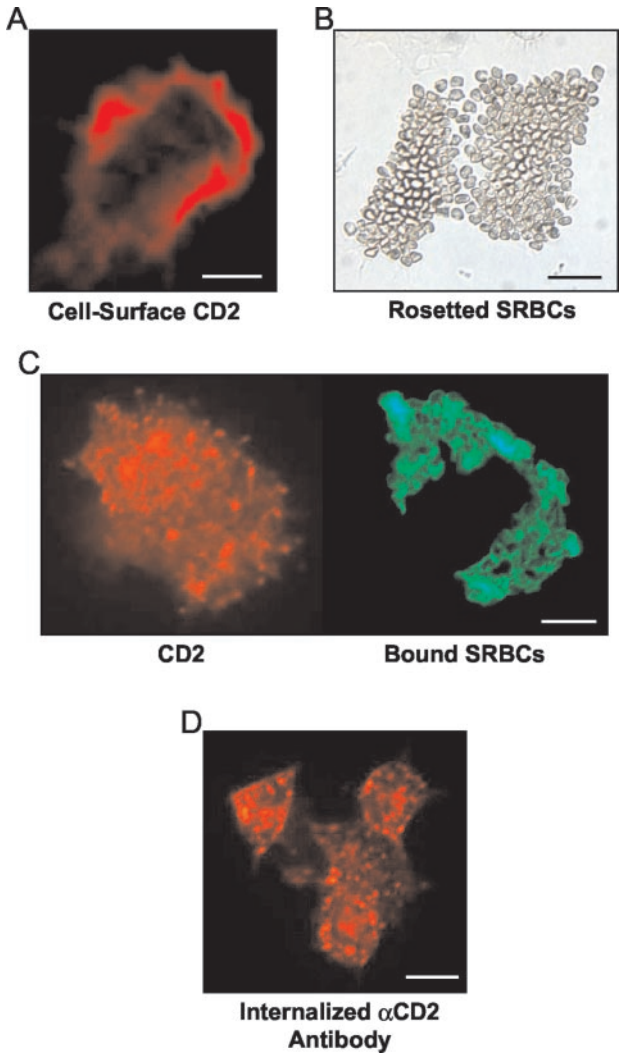

FIG. 5. **Macrophages expressing a CD2-LRP chimeric receptor with a mutated LRP cytoplasmic tail bind but do not internalize SRBCs.** RAW-LR5 macrophages transfected with CD2:LRP:LRP_{mut} were incubated with SRBCs and then assayed for cell surface expression of CD2 (*A*); binding of SRBCs, where an example of rosetting is displayed (*B*); simultaneous expression of CD2 (*red*) and binding of SRBCs (*green*) (*C*); and endocytosis of anti-CD2 antibody (*D*). The *scale bars* in *panels A–C* are 5 μm; the *bar* in *panel D* is 10 μm.

DISCUSSION

Understanding how the LDL receptor and LRP function in the internalization of large particles has implications for fundamentally important biological and pathobiological processes such as macrophage foam cell formation during atherogenesis and the clearance of apoptotic cells. In terms of atherogenesis, the LDL receptor functions in a phagocytic-like manner in the uptake and degradation of large aggregates of LDL by macrophages (Refs. 11, 12, 21, and 22 and Fig. 1), and LRP functions in a similar manner in the uptake of matrix-retained LDL by macrophages (9) and aggregated LDL by smooth muscle cells (10). In view of *in vivo* data showing a role for the LDL receptor in atherogenesis (25, 26), together with the presence of LRP on macrophages and smooth muscle cells in atherosclerotic lesions (27–29) and the presence of both aggregated and retained LDL in lesions (30), it is likely that the phagocytic-like capabilities of these two receptors contribute to foam cell formation during atherogenesis. Regarding the clearance of apoptotic bodies, Henson and co-workers (6, 8) have shown that calreticulin on the surface of macrophages recognizes collectins, such as C1q, mannose-binding lectin, and surfactant proteins A and D, that are bound to apoptotic T cells and neutrophils. Calreticulin then mediates internalization of the opsonized cells by interacting directly with LRP. Thus, the phagocytic-like function of LRP may play a crucial role in the maintenance of the immune system and in the resolution of inflammation.

The key issue addressed in this report was how these two receptors effect their phagocytic-like functions. In particular, we determined whether their cytoplasmic tails alone or in combination with their transmembrane domains could, like known phagocytic receptors (2–5), direct the phagocytosis of RBCs in a chimeric receptor system. In the case of known phagocytic receptors, notably IgG-Fc receptors, the tail domains are substrates for tyrosine kinases, and they recruit signaling proteins that mediate cytoskeletal rearrangement and membrane delivery to the phagocytic cup (1). Although the

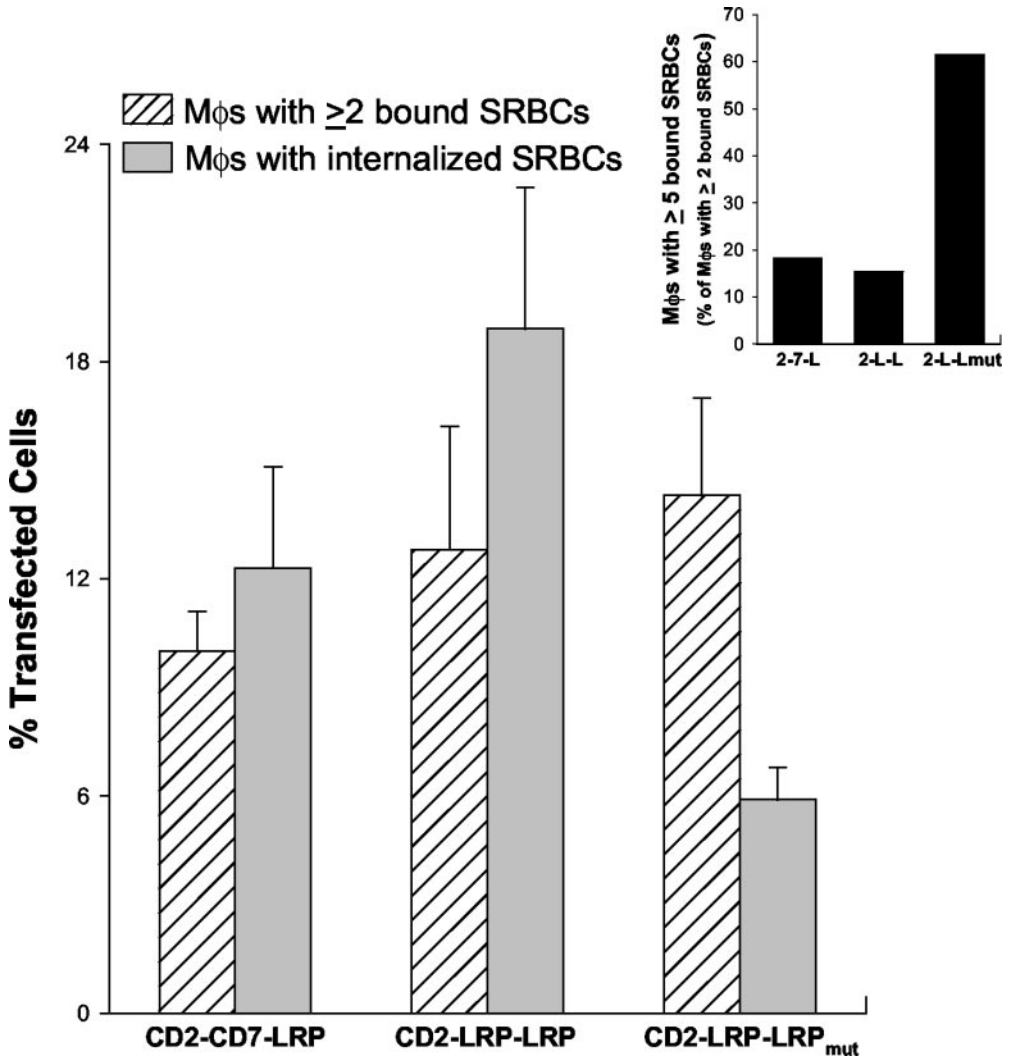

FIG. 6. **Quantification of SRBC binding and internalization in macrophages transfected with LRP-containing chimeric constructs.** The binding and internalization data from the experiment displayed in *Fig. 5* was quantified based upon the observation of three groups of 1000 macrophages for each condition (see Table I for details). *Inset*, quantification of macrophages with ≥ 5 bound SRBCs as a percentage of those cells that bound at least 2 SRBCs.

LDL receptor does not contain phagocytosis-enabling tail motifs found in known phagocytic receptors, such as the ITAM sequence of Fc receptors (1), the LDL receptor tail does contain an NPXY sequence that is necessary for receptor internalization (31) and that likely interacts with the phosphotyrosine-binding domain of two adaptor proteins, the autosomal recessive hypercholesterolemia (ARH) protein and disabled-2 (32, 33). Therefore, these or other tail sequences in the LDL receptor could, in theory, represent novel "phagocytic" motifs (see below). Nonetheless, when subjected to the critical test, neither the LDL receptor tail nor the combination of the LDL receptor transmembrane region and tail were able to direct the phagocytosis of SRBCs by the CD2 ectodomain.

How can these data be reconciled with the finding that the macrophage LDL receptor participates in the internalization of large LDL aggregates by a process that is susceptible to the cytoskeletal and signaling inhibitors described above? One possibility is that the ectodomain of the LDL receptor, which is not present in the chimeric CD2-containing receptor, is necessary for the interaction of the LDL receptor with a phagocytic "co-receptor" on the surface of macrophages (1). A related possibility is that aggregated LDL itself, which is a multivalent ligand, mediates the formation of such a co-receptor complex. Another consideration is the size difference between aggregated LDL (200–500 nm) and SRBCs (8–10 μm). In view of the findings that certain membrane-related phagocytic processes are different depending on whether the particle is large or small (34–39), it is possible that the LDL receptor tail, or tail plus transmembrane region, could direct the cytoskeleton-dependent uptake of particles smaller than RBCs. In this context, Kruth (22) have shown that macrophages sequester aggregated LDL in surface-connected compartments that are narrower than typical phagocytic cups but that nonetheless lead to LDL receptor-mediated particle internalization and lysosomal degradation.

Perhaps the most far-reaching finding in our report is that the LRP cytoplasmic tail is able to direct SRBC phagocytosis in a chimeric receptor system. Despite the previous reports, described above, showing that LRP can mediated the uptake of matrix-retained LDL and apoptotic cells in a phagocytic-like manner, there has been no demonstration that LRP is a phagocytic receptor by the strict criteria used in this study. In our experimental model, we were challenged by the kinetic properties conveyed by the LRP cytoplasmic tail, namely, a low steady-state localization of the receptor on the cell surface. Thus, while a substantial percentage of the CD2-LRP-transfected cells were able to bind a few SRBCs, the type of rosetting observed with the LDL receptor and γ-chain transfectants was not observed. Nonetheless, the transfected macrophages were

clearly able to internalize SRBCs. These data demonstrate the phagocytic capacity of the LRP chimeric receptors, because among thousands of mock- and LDL receptor-transfected macrophages, there was no SRBC internalization.

These new data inspired a preliminary investigation into the signaling motifs in the LRP cytoplasmic tail that may play a role in phagocytosis. There are five recognized domains that have been shown to function in either endocytosis or signal transduction: two NPXY sequences (NPTY and NPVY), two di-leucine motifs, and a Y*XX*L motif (YATL); the second NPXY sequence is in tandem with the Y*XX*L sequence (NPVYATL) (23, 40, 41). We show here that a cytoplasmic tail with mutations in both di-leucine motifs and the tyrosine residue of the NPVYATL sequence results in an increase in cell surface localization of the receptor and in SRBC binding but a 70% decrease in phagocytosis. Interestingly, the mutated chimeric receptor was able to readily endocytose a small, monomeric ligand (*i.e.* anti-CD2 antibody) over a 30-min period, indicating that the receptor could function as an endocytic receptor. Although future experiments will be needed to dissect the exact kinetic properties of the mutated receptor (*e.g.* endocytosis rate, recycling rate, surface:internal ratio), the current data clearly establish that one or a combination of these three tail motifs are important for the phagocytic function of LRP.

Future studies will also be directed at understanding how these tail residues might function in directing phagocytosis. It is possible that one or both of the NPXY sequences play a role similar to that of the NPIY sequence of phagocytic β_1 integrins (42). In this regard, the second NPXY motif (NPVY), the one mutated in CD2:LRP:LRP_{mut}, has been shown to interact with a phosphotyrosine-binding (PTB) domain of a protein called GULP/CED-6, which is involved in the engulfment of apoptotic cells (7). Indeed, this same NP*X*Y motif can be tyrosine-phosphorylated under a number of experimental conditions (40, 41, 43) and is a substrate for tyrosine kinases known to play roles in FcγR-mediated phagocytosis, such as Src, Yes, and Fyn (43). Interestingly, a very similar sequence, with a phenylalanine residue two positions upstream of the asparagine residue (FTNPVY), is found in the LDL receptor tail (FDNPVY), where it binds clathrin heavy chain, clathrin cages, and possibly AP-2 and enables optimal endocytosis (see above and Ref. 44). However, given the inability of the LDL receptor to trigger phagocytosis and the uncertainty surrounding the role of clathrin in phagocytosis (39), it is unlikely that this sequence triggers apoptosis by a mechanism similar to that in clathrin-mediated endocytosis.

The YATL sequence of the LRP tail, which was also mutated in CD2:LRP:LRP_{mut}, is not associated with a another Y*XX*L motif as in the FcγR ITAM sequence, but it is a target for the Src homology 2-(SH2-) containing protein Shc (43). However, another SH2-containing protein, SHIP, has been shown to play a role in down-regulating FcγR-mediated phagocytosis and NFκB signaling (45, 46). The YATL sequence, together with the second di-leucine motif, is also necessary to enable a normal rate of LRP-mediated endocytosis (23). Other proteins that interact with the LRP tail and might play a role in phagocytosis include adaptor-like proteins and signaling molecules, including disabled-1 (Dab1), c-Jun N-terminal kinases (c-Jun), and the scaffold protein FE65 (47). LRP has also been implicated in a variety of downstream cellular signaling pathways, including those involving protein kinase A, and protein kinase C (48, 49). Thus, the Y*XX*L motif is an important sequence to consider in future studies investigating phagocytic triggering by the LRP cytoplasmic tail.

In summary, this report has explored previous suggestions that both the LDL receptor and LRP are phagocytic receptors. By showing that the LDL cytoplasmic tail and transmembrane region are not able to direct phagocytosis of bound SRBCs in a chimeric receptor system, the study focuses attention on other cellular processes related to the phagocytic-like functions of the LDL receptor, such as the participation of co-receptors or alternative cytoskeleton-dependent internalization processes. In contrast, by showing that the cytoplasmic tail of LRP can direct phagocytosis, the study places previous LRP functional studies in a new light and raises important questions as to how LRP coordinates the cytoskeletal and membrane-related signaling reactions that are necessary for phagocytosis. The importance of such future studies is related to the role of the phagocytic-like functions of the LDL receptor and LRP in foam cell formation and atherogenesis and, in the case of LRP, in the process of apoptotic cell clearance.

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