The C-type Lectin Receptor Endo180 Displays Internalization and Recycling Properties Distinct from Other Members of the Mannose Receptor Family*  

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Endo180/urokinase plasminogen activator receptor-associated protein together with the mannose receptor, the phospholipase A₂ receptor, and DEC-205/MR6-gp200 comprise the four members of the mannose receptor family. These receptors have a unique structural composition due to the presence of multiple C-type lectin-like domains within a single polypeptide backbone. In addition, they are all constitutively internalized from the plasma membrane via clathrin-mediated endocytosis and recycled back to the cell surface. Endo180 is a multifunctional receptor displaying Ca²⁺-dependent lectin activity, collagen binding, and association with the urokinase plasminogen activator receptor, and it has a proposed role in extracellular matrix degradation and remodeling. Within their short cytoplasmic domains, all four receptors contain both a conserved tyrosine-based and dihydrophobic-based putative endocytosis motif. Unexpectedly, Endo180 was found to be distinct within the family in that the tyrosine-based motif is not required for efficient delivery to and recycling from early endosomes. By contrast, receptor internalization is completely dependent on the dihydrophobic motif and modulated by a conserved upstream acidic residue. Furthermore, unlike the mannose receptor, Endo180 does not function as a phagocytic receptor in vitro. These findings demonstrate that despite an overall structural similarity, members of this receptor family employ distinct trafficking mechanisms that may reflect important differences in their physiological functions.

Endo180 was first identified as a recycling endocytic receptor expressed in fibroblastic cells (1). Based on peptide sequences obtained from purified protein, a full-length human cDNA clone was isolated, and Endo180 was demonstrated to be the fourth and last member of the mannose receptor family (2–4). In addition to Endo180, the mannose receptor family comprises the mannose receptor, the M-type phospholipase A₂ receptor (PLA₂,R),¹ and DEC-205/MR6-gp200. This family grouping is based on an overall structural conservation with the four recep-tors containing a large extracellular domain comprising an N-terminal signal sequence followed by a cysteine-rich domain, a fibronectin type II domain, and 8 or 10 C-type lectin-like domains (CTLDs). The single pass transmembrane domains are followed by a short cytoplasmic domain. As a family, these receptors have two striking features. First, although they belong to the large C-type lectin superfamily (information available on the World Wide Web at ctld.glycob.ox.ac.uk), they uniquely contain multiple CTLDs within a single polypeptide backbone (4–9). Second, they share the ability to be recycled between the plasma membrane and intracellular compartments of the cell (1, 8, 10, 11).

As a consequence of their structural and recycling characteristics, it was initially assumed that these receptors would commonly function to internalize glycosylated ligands for intracellular delivery. However, further characterization has revealed the following. (a) Although the receptors contain multiple CTLDs, only CTLDs 4 and 5 of the mannose receptor and CTLDs 1 and 2 of Endo180 contain the conserved amino acids found in functional C-type lectins, and accordingly only these two receptors have been demonstrated to exhibit Ca²⁺-dependent sugar binding (2, 12). (b) At least some of the CTLDs in this receptor family have evolved to mediate protein/protein interactions rather than protein/sugar interactions. This is exemplified in the PLA₂,R, which binds nonglycosylated secretory phospholipase A₂ in a Ca²⁺-independent manner via CTLD5 (13). (c) Domains in addition to the CTLDs can mediate ligand interactions. The cysteine-rich domain of the mannose receptor has been demonstrated to bind sulfated sugars, and structural studies and sequence analysis have suggested that this feature is not shared with other family members (4, 14). The fibronectin type II domain of the PLA₂,R has been demonstrated to bind collagen, and similarly structural and sequence analysis predicts that this feature will be shared with other family members with the possible exception of DEC-205. In the case of Endo180, collagen binding both in vitro and in vivo has been demonstrated (3).² (d) In addition to binding soluble ligands, members of this receptor family can bind transmembrane ligands, and this can occur both in cis and trans. For example, the mannose receptor on lymphatic endothelia interacts with leukocyte L-selectin (15), whereas Endo180 was identified independently as part of a trimolecular complex with the urokinase plasminogen activator receptor and pro-urokinase plasminogen activator, hence its alternative name, urokinase plasminogen activator-receptor-associated protein, or uPARAP

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¹ The abbreviations used are: PLA₂,R, phospholipase A₂ receptor; CTLD, C-type lectin-like domain; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorting; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; RFI, relative fluorescence intensity; RPE, R-phycocerythrin.

² D. Wienke and C. M. Isacke, unpublished observations.
(3). (e) In addition to variation in ligand binding properties, these four family members do not share a common intracellular destination. Recently, it has been demonstrated that whereas the mannose receptor is predominantly localized to early endosomes, DEC-205 is targeted to late endosome/lysosomal compartments (16). Moreover, the mannose receptor is unusual in that, in addition to its ability to be internalized via the clathrin-mediated endocytic pathways, it can also mediate phagocytosis of nonopsonized microorganisms or synthetic large particulate ligands (17, 18). Together, these data demonstrate that rather than representing a group of related lectin receptors, the mannose receptor family is a group of multidomain receptors with distinct ligand binding and trafficking properties.

In situ hybridization analysis together with immunohistochemistry has revealed that most tissues have Endo180 expression but that this is generally restricted to stromal cells, macrophages, and a subset of endothelial cells (1, 2, 19). In addition, high levels of expression are found in the embryo and neonate in chondrocytes at areas of active cartilage deposition and in tissues undergoing primary ossification and on chondrocyte (data not shown) and osteoblast (22) cell lines. This distribution pattern together with its C-type lectin activity (2), collagen binding ability, and interaction with urokinase plasminogen activator receptor (3) suggests a role for Endo180 in regulating extracellular matrix degradation and remodeling. In support of this hypothesis is the observed up-regulation of Endo180 on angiogenic endothelial cells (23) and on the stromal fibroblasts and myoepithelial cells in breast tumors (19), where increased expression may be required for the dissolution of basement membranes lining the blood vessels or epithelial sheets and/or for degradation of extracellular matrix components associated with the tumor. Finally, the observed C-type lectin activity of Endo180 and the demonstration that it is expressed on macrophages (2) raises the possibility that like the mannose receptor, Endo180 could function both as an endocytic receptor and as a phagocytic receptor. To understand the physiological role of Endo180, we have undertaken experiments to determine the mechanism by which Endo180 is internalized from the plasma membrane, the intracellular destination of this receptor, and the potential that Endo180 can mediate phagocytosis in addition to endocytosis.

EXPERIMENTAL PROCEDURES

Generation of Endo180 Cytoplasmic Domain Constructs—Cloning and generation of the pcDNA3-Endo180 construct has been described elsewhere (2). In addition, Endo180 was inserted into the NotI and XhoI sites of a pcDNA3 vector in which the HindIII site had been removed (pcDNA3-HindIII). For generation of the cytoplasmic domain mutants, pcDNA3-Endo180 or pcDNA3-HindIII-Endo180 was subject to site-directed mutagenesis using the QuikChange XL kit (Stratagene). The pcDNA3-Endo180(Ala1452) pcDNA3HindIII-Endo180(Ala1469/

RESULTS

Generation and Expression of Endo180 Cytoplasmic Domain Mutants—Examination of the mannose receptor family cytoplasmic domains reveals that all members contain two putative endocytosis motifs, the first based on a conserved tyrosine residue and the second based on a conserved dihydrophobic motif (Fig. 1a). In the mannose receptor, PLA2R, and DEC-205, mutational analysis has indicated that it is the tyrosine-based motif that primarily mediates the internalization of these receptors into the endocytic pathway (10, 11, 16, 25). To determine whether the equivalent motif was responsible for Endo180 internalization, the conserved tyrosine was mutated, pcDNA3-Endo180(Ala1452). In addition, alanine substitutions were made in the conserved Leu-Val dihydrophobic motif (Fig. 1b). Mutations on receptor trafficking have employed chimeric constructs; however, since it was demonstrated for the mannose receptor that different results can be obtained with different chimeras (10), these mutations were made in the context of full-length human Endo180 and Endo180 monitored using a human-specific anti-Endo180 mAb. Wild type and mutant constructs were trans-
fected into murine NIH-3T3 cells as these cells express endogenous Endo180 and therefore will contain the correct trafficking machinery. As has been described by others (26), it was noted in preliminary studies that overexpression of Endo180 in cells in transient transfections resulted in an abnormally high percentage of receptor localized to the plasma membrane (data not shown). Consequently, permanently expressing populations were generated by selecting transfected cells in G418 and enriching for receptor-positive cells by magnetic immunobead sorting. To assess the subcellular localization of Endo180, cells were either stained prior to fixation to determine the cell surface distribution or fixed and then stained to assess the total cellular distribution (Fig. 3). Wild type human Endo180 expressed in murine NIH-3T3 cells showed a distribution indistinguishable from endogenous Endo180 in MG-63 cells. A distinct punctate plasma membrane staining was observed, which has previously been demonstrated to represent clustering of receptors in clathrin-coated pits (1). Intracellularly, a strong vesicular staining was observed, which was concentrated in the perinuclear region. In agreement with the FACS analysis and immunoblotting results (Fig. 2), NIH-3T3 cells transfected with vector alone showed no staining with the anti-human Endo180 mAb (data not shown). Analysis of the Endo180 mutants gave unexpected results. Substitution of the conserved tyrosine residue with an alanine residue, Endo180(Ala1452), resulted in a receptor with a distribution identical to that of wild type Endo180. In contrast, mutation of the dihydrophobic motif, Endo180(Ala1468/ Ala1469), resulted in a highly diffuse Endo180 staining over the whole plasma membrane, and this staining pattern did not substantially change when cells were permeabilized prior to staining. This increased cell surface expression is in keeping with the higher fluorescence intensity observed in the FACS staining of this mutant (Fig. 2a). It has been reported that the presence of an acidic acid residue in the –3 to –4 position to a dihydrophobic motif play a role in the internalization of receptors from the cell surface and/or in regulating their intracellular trafficking (see "Discussion"). Consequently, the distribution of Endo180 containing an alanine substitution for the conserved glutamic acid, Endo180(Ala1468/Ala1469), was examined. Interestingly, this receptor showed a partial phenotype with a stronger, more evenly distributed cell surface staining compared with wild type Endo180 or Endo180(Ala1452) but with stronger intracellular staining than was observed with Endo180(Ala1468/Ala1469). To determine whether this intracellular staining observed with Endo180(Ala1464) and the other mutants resulted from internalization of receptor from the cell surface, the trafficking of the receptor was monitored using 125I-anti-Endo180 mAb.

MG-63 cells or NIH-3T3 cells expressing the different constructs were incubated on ice with 125I-anti-Endo180 mAb, washed, and then warmed to 37 °C for 0–30 min. At each time point, the cells were incubated with an acid strip pH 2.5 buffer to determine the amount of cell surface-associated radioactivity, and the cells were then trypsinized and counted to assess the amount of internalized antibody (Fig. 4). In control experiments, it was determined that acid stripping removed >95% of cell surface-associated antibody, that vector alone-transfected cells showed background binding of the 125I-anti-Endo180 mAb.
that was <10% of that bound to cells expressing wild type Endo180, and that the binding and internalization of \(^{125}\text{I}\)-E1/183 was identical to that of \(^{125}\text{I}\)-E1/183 Fab' fragments (1) (and data not shown). A comparison of MG-63 cells and NIH-3T3 cells transfected with wild type human Endo180 revealed a similar internalization profile. \(^{125}\text{I}\)-anti-Endo180 mAb was
Endo180(Ala1464) were either cell surface-stained by incubating for 1 h with 125I-anti-Endo180 mAb at 37 °C, thereby detecting on the cell surface and 84% intracellularly. Again a similar profile was observed with NIH-3T3 cells expressing wild type Endo180 and Endo180(Ala1452). In contrast, in cells expressing Endo180(Ala1469), only 33% was detected inside the cell, whereas 67% was retained on the plasma membrane. In cells expressing Endo180(Ala1464), some variability in distribution was found between experiments, but an increase in cell surface expression was observed compared with endogenously expressed and transfected wild type Endo180.

Together these data indicate that Endo180 is unique among the mannose receptor family in that mutation of the conserved cytoplasmic tyrosine residue does not affect its distribution or trafficking. In contrast, substitution of the dihydrophobic Leu-Val motif at position 1468/1469 results in a near total restriction of Endo180 to the cell surface, thereby effectively removing it from the endocytic system. As has been demonstrated for other receptors that utilize a dihydrophobic endocytosis motif, the presence of an upstream acidic acid residues plays a role in modulating Endo180 trafficking. Substitution of this residue results in a receptor that has a more diffuse distribution at the cell surface and a reduced rate of internalization. However, after 90 min of incubation with 125I-anti-Endo180 mAb, a substantial proportion of receptor is found intracellularly.

**Endo180 Is Localized to the Early Endosomes**—In previous immunoelectron microscopy and immunofluorescence analyses, Endo180 was demonstrated to be clustered on the cell surface in clathrin-coated pits (1). In permeabilized cells, it was noted that the distribution of Endo180 was similar to that of the transferrin receptor, although in these studies direct colocalization was not assessed. Recently, it has been demonstrated that other members of the mannose receptor family target to distinct intracellular destinations; in particular, the mannose receptor has been shown to predominantly localize to early endosomes, whereas DEC-205 is found in the late endosomes/lysosomes (see “Discussion”). Consequently, it was important to better determine the intracellular localization of wild type Endo180 and to assess whether Endo180(Ala1464), which showed a partial impairment in internalization, was nevertheless able to be delivered to the correct intracellular compartment. To address these issues, MG-63 cells were double-labeled with antibodies directed against Endo180 and either the transferrin receptor as a marker of early endosomes or LAMP-1 as a marker of late endosomes/lysosomes (see “Discussion”). Consequently, it was important to better determine the intracellular localization of wild type Endo180 and to assess whether Endo180(Ala1464), which showed a partial impairment in internalization, was nevertheless able to be delivered to the correct intracellular compartment. To address these issues, MG-63 cells were double-labeled with antibodies directed against Endo180 and either the transferrin receptor as a marker of early endosomes or LAMP-1 as a marker of late endosomes/lysosomes (see “Discussion”). Consequently, it was important to better determine the intracellular localization of wild type Endo180 and to assess whether Endo180(Ala1464), which showed a partial impairment in internalization, was nevertheless able to be delivered to the correct intracellular compartment. To address these issues, MG-63 cells were double-labeled with antibodies directed against Endo180 and either the transferrin receptor as a marker of early endosomes or LAMP-1 as a marker of late endosomes/lysosomes (see “Discussion”). Consequently, it was important to better determine the intracellular localization of wild type Endo180 and to assess whether Endo180(Ala1464), which showed a partial impairment in internalization, was nevertheless able to be delivered to the correct intracellular compartment.

**FIG. 3.** Subcellular distribution of Endo180 cytoplasmic domain mutants. MG-63 cells or NIH-3T3 cells expressing wild type (WT) Endo180, Endo180(Ala1452), Endo180(Ala1468/Ala1469), or Endo180(Ala1464) were either cell surface-stained by incubating for 1 h at 4 °C with anti-Endo180 mAb A5/158; fixed, permeabilized, and stained with Alexa 488 anti-mouse Ig (– saponin); or fixed, permeabilized with 0.2% saponin, and stained with mAb A5/158 followed by Alexa 488 anti-mouse Ig and nuclei counterstained with TOPRO-3 (+ saponin). Scale bar, 20 μm.

very rapidly internalized from the cell surface upon warming to 37 °C, with 64 and 74% of cell surface-bound antibody found cell-associated within 2 min, respectively (Fig. 4). After 10 min, only 10–15% of receptor was detected on the cell surface (Fig. 4). An essentially identical profile was obtained with cells expressing the Endo180(Ala1452) mutant, again indicating that mutation of the conserved tyrosine residue does not impair interaction with the endocytic internalization machinery. In cells expressing Endo180(Ala1468/Ala1469), antibody internalization was drastically impaired, with less than 15% of receptor internalized within the first 2 min and 78% of the receptor still detected at the cell surface after 30 min of incubation at 37 °C. Mutation of the acidic residue upstream of the dihydrophobic motif, Endo180(Ala1464), resulted in a partial phenotype in that antibody could be internalized but at a much reduced rate compared with wild type Endo180 or Endo180(Ala1452) with 28% internalized within the first 2 min after warming. In addition to this slower rate of internalization, the total amount of receptor internalized was reduced such that after a 30-min incubation at 37 °C, the amount of receptor within the cells was less than 50%.

To assess the steady state distribution of Endo180 between the plasma and intracellular membranes, cells were incubated with 125I-anti-Endo180 mAb at 37 °C for 1.5 h, and then the proportion of cell surface and intracellular antibody was assessed (Fig. 5). In MG-63 cells, 16% of the counts were detected on the cell surface and 84% intracellularly. Again a similar profile was observed with NIH-3T3 cells expressing wild type Endo180 and Endo180(Ala1452). In contrast, in cells expressing Endo180(Ala1469), only 33% was detected inside the cell, whereas 67% was retained on the plasma membrane. In cells expressing Endo180(Ala1464), some variability in distribution was found between experiments, but an increase in cell surface expression was observed compared with endogenously expressed and transfected wild type Endo180.
Given the predominant cell surface localization of the Endo180(Ala1468/Ala1469) receptor, essentially no overlap between this mutant and the transferrin receptor was observed. Of particular interest was the intracellular localization of the Endo180(Ala1464) mutant, since this mutant has a diffuse plasma membrane distribution but can be internalized albeit inefficiently. A considerable overlap in the localization of this intracellular population with the transferrin receptor was observed, suggesting that mutation of the acidic residues results in a defect in recruitment into the clathrin-coated pits rather than in delivery to or recycling from the early endosomes.

Endo180 as a Phagocytic Receptor—A striking feature of the mannose receptor is its ability to mediate both clathrin-dependent internalization of ligands into the endosomal system and phagocytic internalization of nonopsonized microorganisms or ligand-coated synthetic particles (17, 18). Moreover, the efficiency of both of these internalization processes is severely impaired by mutation of the conserved cytoplasmic tyrosine residue (10). To investigate whether Endo180 functioned as a phagocytic receptor and, if so, whether this was dependent on a key motif(s) within the cytoplasmic domain, phagocytic assays were performed with NIH-3T3 cells expressing human Endo180. Fibroblasts are not professional phagocytes, but, like the COS cells used for the mannose receptor studies, they can act as nonprofessional phagocytes (27) (e.g. to internalize collagen (28) and apoptotic cells (29)). To investigate the suitability of these cells for phagocytic assays, they were transiently transfected with the FcγIIA (CD32) receptor and tested for their ability to internalize anti-Endo180 mAb-coated 1-μm polystyrene FITC beads. FcγIIA receptors bind to the Fc domain of immunoglobulins to mediate phagocytic uptake of opsonized microorganisms (30, 31), and this can be monitored by FACS analysis (32) and confocal microscopy. For FACS analysis (Fig.

![Equilibrium distribution of Endo180.](image)

**Fig. 4.** Trafficking of Endo180 cytoplasmic domain mutants. MG-63 cells (a) or NIH-3T3 cells expressing wild type (WT) Endo180 (b), Endo180(Ala1464) (c), or Endo180(Ala1468/Ala1469) (d) were incubated on ice for 1 h with 125I-anti-Endo180 mAb E1/183, washed, and then incubated in binding buffer at 37 °C for 0–30 min. At each time point, the amount of 125I-E1/183 in the incubation medium (not shown), acid-dissociable from the cell surface (black lines and circles), or remaining cell-associated (gray lines and squares) was collected and counted. f, the percentage of 125I-E1/183 internalized after a 2-min incubation at 37 °C. Values given are for duplicate samples, with error bars showing S.D. values. Similar results were obtained on three separate occasions.

![Equilibrium distribution of Endo180.](image)

**Fig. 5.** Equilibrium distribution of Endo180. Cells plated overnight in 35-mm dishes were incubated with 125I-anti-Endo180 mAb E1/183 for 90 min at 37 °C. Cells were then washed extensively, and the amount of 125I-E1/183 dissociable from the cell surface by acid stripping (dark bars) and remaining cell-associated (pale bars) was assessed. Values given are for duplicate samples, with error bars showing S.D. values. Similar results were obtained on three separate occasions. WT, wild type.
cells that had been incubated with mAb- or BSA-coated FITC beads for 4 h at 37 °C were washed, and cell surface-associated beads were removed by acid stripping as described for the endocytosis assays. A comparison of FcγIIA receptor or mock-transfected cells revealed that (a) as expected for a transient transfection, only a proportion of the FcγIIA receptor-transfected cells were associated with FITC beads, (b) in receptor-transfected cells, cells that were associated with beads were observed as a wide peak, suggesting that variable numbers of beads had been internalized, (c) the fraction of bead-positive cells and the fluorescent intensity of positive cells was much reduced when FcγIIA receptor cells were incubated with BSA beads and similarly when mock-transfected cells were incubated with either mAb beads or BSA beads. To facilitate further analysis, a reference system was required so that the phagocytic ability of both transiently and permanently transfected cells expressing different receptors could be compared. For this purpose, the ratios of the mean fluorescence intensities of cells incubated with beads to cells incubated without beads were calculated to give a relative fluorescence intensity (RFI). A phagocytic index for the receptors under investigation was generated by dividing the RFI values for cells expressing receptor with nonexpressing or mock-transfected cells. The RFI values for cells transfected with FcγIIA receptor or mock-transfected and incubated with mAb-coated FITC beads were 13.35 and 2.45, respectively, giving a phagocytic index of 5.26. The RFI values of the same cells incubated with BSA beads were 2.57 and 3.60, respectively, giving a phagocytic index of 0.71. To confirm that within the transiently transfected populations, mAb-coated beads specifically associated with FcγIIA receptor-expressing cells, parallel experiments were undertaken with cells stained prior to FACS analysis with RPE-conjugated anti-FcγIIA receptor (RPE-anti-CD32) antibody (Fig. 8b). By comparing cells incubated with and without anti-CD32 mAb and with or without either mAb- or BSA-coated FITC beads, it was demonstrated that cells expressing FcγIIA receptor were selectively associated with mAb-coated FITC beads. Finally, although FACS analysis demonstrates a specific association of mAb beads with FcγIIA receptor-expressing cells, it cannot distinguish between beads that have been internalized and cell surface-associated beads that have not been removed by acid stripping. Consequently, confocal microscopy studies were undertaken on cells in which the acid strip to remove cell surface-associated beads was omitted, and cells were stained after fixation with mAb IM7 and the nuclei were counterstained with TOPRO-3 (Fig. 9). mAb IM7 recognizes murine CD44, an abundant transmembrane protein that is predominantly restricted to the plasma membrane (33). Analysis of FCγIIA receptor-transfected cells incubated with mAb-coated FITC beads revealed the presence of multiple FITC beads associated with the cells that by z-sectional analysis were clearly located intracellularly. By contrast, few BSA-coated FITC beads were associated with the cells, and these were predominantly cell surface-associated. Similarly, few mAb-coated or BSA-coated beads were...
FIG. 8. FcγIIA receptor can mediate phagocytosis in NIH-3T3 cells. NIH-3T3 cells were transiently transfected with the pRK5-FcγIIA plasmid or mock-transfected. 24 h later, cells were incubated in serum-free medium for 1 h and then for 4 h with mAb-coated FITC beads, BSA-coated FITC beads, or no beads. Cells were washed in PBS, incubated in ice-cold pH 2.5 acid wash for 5 min to remove cell surface-associated beads, washed in PBS, detached with trypsin, and fixed in 1% paraformaldehyde. a, cells incubated with (solid profiles) or without (open profiles) FITC beads were subject to single-channel FACS analysis. b, cells treated as described above were incubated with or without RPE-conjugated anti-CD32 (anti-FcγIIA receptor) antibody and subjected to two-color FACS analysis. Quadrants were set on cells incubated without FITC beads and without RPE-anti-CD32 antibody.
FITC beads were associated with mock-transfected cells. Together, these data demonstrate that NIH-3T3 cells are capable of mediating uptake of ligand coated synthetic beads, and, as a consequence, they were judged a suitable in vitro system for analyzing the phagocytic properties of Endo180.

To monitor phagocytic uptake by Endo180, the same anti-human Endo180 mAb-coated FITC beads were employed, since these will specifically associate with cells expressing transfected human Endo180 but not with endogenous murine Endo180. As expected, a similar FACS profile was obtained for Endo180 expressing NIH-3T3 cells and parental NIH-3T3 cells incubated with BSA-coated FITC beads (Fig. 10a). The RFI values for these two cell types were 4.68 and 4.71, respectively, giving a phagocytic index of 0.99. In contrast to cells transiently transfected with the FcγIIA receptor, the FACS profile of NIH-3T3 cells expressing Endo180 incubated with mAb-coated FITC beads was similar to that of parental NIH-3T3 cells, with RFI values of 2.55 and 4.71, respectively, giving a phagocytic index of 0.54. Confocal microscopy analysis (Fig. 10b) revealed that in the absence of acid stripping, there was an increased number of mAb-coated beads, compared with BSA-coated beads, associated with wild type Endo180-transfected cells. However, by z section analysis, these beads were found predominantly associated with the cell surface, and few were detected intracellularly. As previously described for mock-transfected cells (Fig. 9), few mAb- or BSA-coated beads were found associated with parental cells (data not shown). To confirm that this result was not due to an intrinsic defect in the transfected NIH-3T3 cells, phagocytosis experiments were repeated with MG-63 cells. Essentially identical results were obtained. Transfection with the FcγIIA receptor resulted in uptake of mAb beads but not BSA beads with phagocytic indices of 2.85 and 0.41, respectively (data not shown). In nontransfected cells, there was no uptake of either mAb or BSA beads (Fig. 10a; RFI values of 2.69 and 2.83, respectively) despite endogenous expression of human Endo180 (Fig. 2). These data indicate that the FcγIIA receptor, but not the Endo180 receptor, is capable of efficient phagocytic internalization of ligand-coated synthetic particles.

DISCUSSION

Clathrin-mediated Endocytosis—A characteristic feature of the four mannose receptor family members is that they are all subject to constitutive clathrin-mediated endocytosis. For Endo180, examination of the kinetics has revealed this to be a rapid process, with 64–70% of cell surface receptor being in-
Clathrin-mediated internalization requires a “signal” within the cytoplasmic domain that allows association of the transmembrane proteins with adaptor complexes, in particular with the plasma membrane-localized AP-2 complexes (34–36). An examination of the cytoplasmic domains of the mannose receptor family demonstrates two potential endocytosis motifs (Fig. 1). The first is based on a conserved tyrosine within a low density lipoprotein receptor type motif, $\phi X N X X Y$, in which $\phi$ represents a bulky hydrophobic residue (34). This motif is well conserved within the man-
nose receptor and, interestingly, conserved in some PLA$_2$R and DEC-205 species but not others. Despite these differences, mutation of the conserved tyrosine residue within the mannose receptor, PLA$_2$R, and DEC-205 severely impairs receptor internalization. For PLA$_2$R, this mutant is essentially internalization-deficient (11), whereas in DEC-205 internalization is reduced to 50% of wild type values (16). Initially, it was reported that a mannose receptor tyrosine mutant had a similar phenotype to DEC-205 (10). However, more recently it has been reported that this conserved tyrosine is essential for mannose receptor endocytosis (25). In contrast to these other family members, we show here that mutation of the conserved tyrosine in Endo180 has no effect on receptor internalization or intracellular destination. In addition to the tyrosine-based motif, all members of the mannose receptor family have a dihydrophobic sequence of LV, LM, LI, or ML (Fig. 1). Dihydrophobic based endocytosis motifs have been identified in a number of receptors including the Fc receptor, major histocompatibility complex class II-associated invariant chains, mannose 6-phosphate receptors, and IFN$\gamma$ receptor (34–37). Mutation of the dihydrophobic Leu-Val amino acids in Endo180 essentially blocks receptor internalization. Among constitutively endocytosed receptors with dihydrophobic motifs, Endo180 is somewhat unusual in that it is targeted from the plasma membrane to the recycling endosomes rather than from the cell surface or trans-Golgi network to a late endosome/lysosome compart-ment, suggesting that other motifs or the sequence context of the dihydrophobic motif provides additional targeting specificity. One such candidate is the upstream acidic residue, Asp$^{1464}$, which is conserved in all mannose receptor family members (Fig. 1a). An acidic acid residue in the −3 or −4 position has been subjected to close scrutiny in other receptors (35, 38, 39) and has been shown to be required for intracellular trafficking from the early endosome and in some cases to modulate internalization. However, mutation of this residue in Endo180, although resulting in impaired internalization, does not result in receptor mistargeting (see below).

In other members of the mannose receptor family, the role of the dihydrophobic motif has not been so extensively examined. In the PLA$_2$R, mutation of the leucine within the Leu-Ile motif to a glycine had no effect on receptor internalization (11). In DEC-205, generation of a truncated cytoplasmic domain that retains the conserved tyrosine motif but removes the dihydrophobic motif does not affect the rate of receptor internalization (16). Both tyrosine-based and dihydrophobic motifs interact with the adaptor complex AP2 although most likely at separate binding sites/subunits within the complex (35, 36), which raises the interesting issue of why members of this receptor family that are efficiently endocytosed from the cell surface contain two putative endocytosis motifs but only utilize one, and why different family members utilize different motifs.

**Trafficking of Receptor from the Early Endosomes**—Despite the rapid rate of Endo180 endocytosis, at steady state 15–25% of the total receptor population is located at the cell surface (Fig. 5), and the receptor has a long half-life of ~24 h (1), indicating that Endo180 is efficiently recycled back to the cell surface from intracellular compartments. Once recruited into clathrin-coated pits, receptors are internalized into early endosomes. At this stage, segregation occurs, targeting some proteins into recycling vesicles destined for return to the plasma membrane, whereas others are destined for vesicular transport to the late endosomes/lysosomes (34, 40). Recent detailed examination of the mannose receptor family has revealed that the mannose receptor (16, 41) and Endo180 (Figs. 6 and 7) are predominantly found in early endosomes, raising the question of whether recycling from this compartment is a signal-depend-ent or signal-independent mechanism. For the mannose receptor, this has been addressed by generating receptor chimeras with an additional C-terminal endocytosis motif that can rescue the internalization defect associated with mutation of the conserved tyrosine residue (25). However, in these chimeric proteins, mutation of this conserved tyrosine residue together with the adjacent phenylalanine results in a receptor that is efficiently internalized but mistargeted to the late endosomes/lysosomes, indicating that the diaromatic Tyr-Phe motif is required for recycling from the early endosome to the plasma membrane. A diaromatic residue Tyr-Pyr is present in the PLA$_2$R, but neither the intracellular destination of this receptor nor the ability of this motif to mediate recycling has yet been investigated. DEC-205 does not contain an equivalent diaromatic motif, and this is in keeping with the recent demonstration that this receptor is not recycled from the early endosomes but rather is localized to the late endosome/lysosomes (16). As discussed above, truncation of DEC-205 C-terminal to the conserved tyrosine residue results in a receptor that is efficiently internalized. However, this truncated DEC-205, instead of being delivered to the late endosomes/lysosomes, is colocalized with the transferrin receptor in the early endosomes. Moreover, these receptors showed inefficient antigen presentation, demonstrating a functional requirement for DEC-205 to be delivered to the degradative compartments. Further mutagenesis identified three acidic residues, EDE, as a late endosome/lysosomal targeting motif (Fig. 1) (16). Unlike the mannose receptor and the PLA$_2$R, Endo180 does not contain a diaromatic motif, yet it is efficiently recycled from the early endosome. It may be that since Endo180 does not utilize a tyrosine-based motif for recruitment into the clathrin-coated pits, it employs a separate recycling motif. In addition, it has been suggested that association of recycling receptors with specific membrane lipid domains has a role to play in segrega-tion from the late endosomal/lysosomal pathway (40). For the mannose receptor family, association with membrane lipids either directly or via linker proteins has not been investigated. However, swapping of the mannose receptor transmembrane domain does impair receptor internalization (10), indicating that this domain may have a role to play in regulating receptor trafficking.

**Phagocytosis**—In mammals, phagocytosis is the process primarily used to engulf microorganisms and apoptotic or unwanted cells and plays a critical role in innate immunity and tissue remodeling (31, 42–44). In addition to the size of the material internalized, the processes of clathrin-mediated endocytosis and phagocytosis are mechanistically distinct. Engagement of phagocytic receptors such as the Fc and complement receptors results in localized polymerization of actin and extension of the membrane to engulf the particle into an intracellular phagosome, which rapidly fuses with the endosomes and/or lysosomes to expose the contents to the hydrolytic enzymes. In this respect, the mannose receptor is unusual in that it can mediate the endocytic clearance of glycosylated ligands and the phagocytic uptake of a wide variety of microorganisms (18).

It would seem unlikely that the PLA$_2$R and DEC-205 would function as phagocytic receptors, given their defined functions in the uptake of macromolecules and their lack of C-type lectin activity. In contrast, Endo180 is expressed both on professional phagocytic cells such as macrophages and nonprofessional phagocytic cells in the stroma and is a functional C-type lectin (2), leading to the suggestion that Endo180 may play a com-plementary or overlapping phagocytic role to the mannose receptor. However, as demonstrated here, although cells expressing Endo180 can bind to 1-μm anti-Endo180 mAb coated beads,
but not control beads, no phagocytic uptake was observed in a system where phagocytic uptake of the same beads by the FcyIIA receptor was readily apparent. These data indicate that Endo180 does not function as a primary phagocytic receptor and, unlike the mannos receptor, cannot mediate uptake of ligand-coated synthetic particles. It remains to be determined whether Endo180, like some integrins, the lipopolysaccharide receptor, and scavenger receptors, may alone not be competent to mediate phagocytosis but may have a role in professional phagocytes in tethering particles and engaging a classical phagocytic receptor to drive the phagocytic machinery (45).

In conclusion, the mannose receptor and Endo180 share a common structural organization and bind a discrete but overlapping set of ligands. In addition, they both constitutively express the Iga receptor to drive the phagocytic machinery (45). To mediate phagocytosis but may have a role in professional phagocytes, indicating a major role in transport between subcellular compartments, and engaging a classical phagocytic receptor to drive the phagocytic machinery (45).

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