

Phagocytic Chimeric Receptors Require Both Transmembrane and Cytoplasmic Domains from the Mannose Receptor

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Summary

Phagocytosis has traditionally been viewed as a specialized function of myeloid and monocytic cells. The mannose receptor (MR) is an opsonin-independent phagocytic receptor expressed on tissue macrophages. When human MR cDNA is transfected into Cos cells, these usually non-phagocytic cells express cell surface MR and bind and ingest MR ligands such as zymosan, yeast, and *Pneumocystis carinii*. Expression of cDNA for FcγRI (CD64), the high-affinity Fc receptor, in Cos cells confers binding but barely detectable phagocytosis of antibody-opsonized erythrocytes (EA). We report here that chimeric receptors containing the ligand-binding ectodomain of the Fc receptor and the transmembrane and cytoplasmic domains of the MR ingest bound EA very efficiently, whereas chimeras with the Fc receptor ecto- and transmembrane domains and the MR tail, or the Fc receptor ecto- and cytoplasmic domains and the MR transmembrane region, are significantly less phagocytic. All of the chimeric receptors bind ligand with equal avidity, but gain of functional phagocytosis is only conferred by the MR transmembrane and cytoplasmic domains. Endocytosis of monomeric immunoglobulin G by chimeric receptors demonstrates a similar pattern, with optimal uptake by the chimera containing both tail and transmembrane regions from the MR. The chimeric receptors with only the transmembrane or the cytoplasmic domain contributed by the MR were less efficient. Site-directed mutagenesis of the single tyrosine residue in the cytoplasmic tail (which is present in a motif homologous to an endocytosis consensus motif in the LDL receptor cytoplasmic tail [Chen, W.-J., J. L. Goldstein, and M. S. Brown. 1990. *J. Biol. Chem.* 265:3116]) reduces the efficiency of phagocytosis and endocytosis to a similar extent.

The recognition, engagement, and internalization of pathogens is a hallmark of myeloid cell function. PMN and mononuclear phagocytes possess at least three classes of phagocytic receptors. The receptors for the Fc portion of Ig and for complement require their respective opsonins in order to recognize pathogens (1). The macrophage mannose receptor (MR),¹ however, recognizes pathogens directly by interaction with various configurations of mannose, *N*-acetylgluco-

samine, and other sugars that are ubiquitously expressed on the surface of pathogens (2, 3). MR expression appears to be restricted to tissue macrophages. Purification of MR from rabbit macrophages and human placenta revealed a glycoprotein of 175 kD (reviewed in reference 2). Characterization of full-length cDNAs from human placenta and macrophage revealed that the encoded protein has a cysteine-rich NH₂ terminus, a fibronectin type II domain, eight carbohydrate recognition-like domains, a single hydrophobic transmembrane region, and a 45-residue cytoplasmic tail (4, 5).

Transient expression of the MR on the surface of Cos1 cells resulted in uptake of soluble mannose-rich glycoconjugates as expected (4). Surprisingly, transfected cells also bound and ingested yeast particles (4). Receptor-ligand interactions appear to occur sequentially around the full cir-

¹ Abbreviations used in this paper: EA, antibody-opsonized erythrocyte; IM, incubation medium; MR, mannose receptor.

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cumference of the target, consistent with the "zipper" model of phagocytosis proposed by Griffin et al. (6). Mutant MR that lack the cytoplasmic tail were expressed on the cell surface, but were unable to ingest bound particles (4). These experiments implied that ligation of the MR by a multivalent ligand is able to signal phagocytosis and what appears to be correct routing to phagolysosomes, even in heterologous cells. By contrast, transfection of various isoforms of Fc γ RI and Fc γ RIII (7, 8) into heterologous cells resulted in transfectants that were able to bind but not ingest antibody-coated particles. The human Fc γ RIIA and murine Fc γ RII-B2 have been shown to mediate phagocytosis of opsonized targets when expressed in heterologous cells (7, 9).

The inability of Fc γ RI, the high-affinity Fc receptor, to internalize bound targets when expressed in heterologous cells provided an opportunity to construct chimeric receptors in which the Fc receptor ectodomain was spliced onto the transmembrane and cytoplasmic domains of the MR. This chimeric receptor was able to phagocytose antibody-coated erythrocytes when transiently expressed in Cos cells. Additional chimeric receptor constructs indicate that both transmembrane and cytoplasmic domains of MR are required to fully rescue the phagocytic phenotype. Endocytosis of monomeric IgG1 by the chimeric constructs showed a similar dependence on the presence of both transmembrane and cytoplasmic domains.

Cytoplasmic tyrosine residues have been shown to be crucial to the endocytic function of a number of receptors (10–14). These critical tyrosines have been suggested to define an endocytic motif by virtue of their propensity to nucleate tight turns in the protein's tertiary structure (11, 12). Mutation of the single cytoplasmic tyrosine in the MR reduces phagocytosis of opsonized erythrocytes and monomeric IgG to a similar modest degree, suggesting that while this tyrosine is not essential to either process in the MR, it may play a role in both processes.

Materials and Methods

Construction of Chimeric Receptor DNAs. All chimeric receptors contained the ectodomain of Fc γ RI (CD64). The Fc γ RI transmembrane region and cytoplasmic tail were replaced by the analogous domains from MR (FMM)², or only the MR tail (FFM), or the transmembrane regions were exchanged (FMF). Complementary oligonucleotides that precisely defined the domain boundaries were synthesized and allowed amplification of each respective domain by PCR. Chimeric FMM containing the Tyr¹⁴¹¹→Ala mutation (numbered as in the MR) was prepared by a PCR-based strategy in which GCT (coding for Ala) replaced TAT (coding for Tyr in the wild-type MR tail) in specific complementary oligonucleotides that spanned this region. Fragments were gel purified and

² The Fc receptor–MR chimeric receptors are denoted by a three-letter code in which the first letter represents the (ligand-binding) ectodomain, the second represents the hydrophobic putative transmembrane region, and the third represents the cytoplasmic tail. F denotes Fc γ RI, and M denotes the mannose receptor.

subcloned into the original expression vector (π H3M, provided by B. Seed, Massachusetts General Hospital, Boston, MA [15]). Constructs were verified by double-stranded dideoxy sequencing (Sequenase; U.S. Biochemical Corp., Cleveland, OH). Plasmid DNA was purified by CsCl banding before transfection (16).

Transfection. Constructs were expressed by transfection into Cos7 cells using DEAE-dextran (15, 16). Cells were transfected with 1 μ g of DNA/75-cm² 75% confluent cells on day 1. On day 2, the cells were trypsinized and replated (including one plate of each construct to assay cell surface receptor expression; see below). On day 3, the assay was performed. Controls included mock-transfected cells, which were processed as transfected cells with the omission of DNA during the transfection step.

Assay for Cell Surface Expression. Expression of the different constructs was variable from one transfection to another. Thus, cell surface expression of receptors was assessed by flow cytometry. Cells were detached from the substrate with PBS/5 mM EDTA and washed by low-speed centrifugation twice. Cells were blocked with PBS/1% BSA, stained with mAb 32.2 to the extracellular domain of Fc γ RI (1:200 dilution; Medarex, West Lebanon, NH), and then with FITC-labeled goat anti-mouse antiserum (1:1,500 dilution), each for 30 min at room temperature, with three washes after each step except the last, which was followed by six washes. Cells were assayed on a Profile 2 flow cytometer (Coulter Electronics, Hialeah, FL). Gates were set based on controls omitting specific antibody and on mock-transfected cells. Phagocytic indices (number of intracellular EA per 100 cells) were divided by the percentage of positive cells relative to Fc γ RI, which was set at 100%, to normalize for differing levels of receptor expression.

Phagocytosis Assay. Human Rh⁺ erythrocytes (E) were washed three times in PBS and opsonized by incubation for 30 min at 37°C with RhoGAM anti-D antibody (1:200 in PBS; Ortho Diagnostic Systems Inc., Westwood, MA). The opsonized erythrocytes (EA) were washed three times more with PBS. Transfected or mock-transfected Cos cells were washed three times with PBS and placed in 5 ml of DME (Gibco Laboratories, Grand Island, NY) containing opsonized RBC at 0.025% hematocrit, and incubated for 1 h at 37°C in 5% CO₂. During phagocytosis, some of the Cos cells (which are normally adherent) detached from the substrate. These were usually cells which had phagocytosed several particles. At the end of the incubation, the medium containing detached cells was collected and reserved on ice. The adherent cells were detached by incubation in PBS/5 mM EDTA followed by vigorous pipetting, and pooled with the reserved medium. The cells were centrifuged, the supernatant removed, and cells were resuspended with gentle pipetting in distilled deionized water for 15–20 s to lyse extracellular EA. An equal volume of 2 \times PBS was added to restore isotonicity, and the cells were diluted in 10 vol of PBS and washed once. They were resuspended in PBS with 1% BSA and the final cell suspension was cytocentrifuged. The slides were stained with Wright's stain and counterstained with Giemsa. At least 100 cells were scored per slide, and duplicate slides were prepared for each condition. The number of intracellular EA was noted for each cell counted. The hypotonic lysis was 99% effective, as judged by controls using untransfected Cos cells and allowing no time for phagocytosis.

Electron Microscopy. Cells were transfected and incubated with EA (without hypotonic lysis) as described above. The cell pellet was then fixed overnight with 2% glutaraldehyde in 0.17 M sodium cacodylate buffer (pH 7.4), treated with 1% OsO₄ for 1 h, stained en bloc with uranyl acetate for 30 min, and then dehydrated. Pellets were then embedded in Epox 812. 75-nm sections were cut, mounted on copper grids, and stained with uranyl acetate and lead

citrate. Sections were examined and photographed on an electron microscope (300; Phillips Electronic Instruments, Inc., Mahwah, NJ).

¹²⁵I-IgG1 Binding. IgG1 (Calbiochem-Behring Corp., La Jolla, CA) was iodinated by the Chloramine-T method as described (4), yielding specific activities of 200 $\mu\text{Ci}/\mu\text{g}$. Transfected cells in 24-well tissue culture plates (Corning Glass Works, Corning, NY) were preincubated in incubation medium (IM) (Eagle's MEM with 0.1% OVA (Sigma Chemical Co., St. Louis, MO) and 20 mM HEPES, pH 7.4) for 1 h at 37°C. They were then placed on ice, rinsed thrice with cold PBS, and placed in 0.3 ml cold IM containing 20 nM ¹²⁵I-IgG1 with varying amounts of added unlabeled IgG1. After incubation at 4°C for 2 h, each well was rinsed four times with cold PBS, solubilized in 1 ml of 0.1 N NaOH, counted, and assayed for total protein (Bio-Rad Laboratories, Richmond, CA). All binding is corrected for total protein. Nonspecific binding determined using a 100-fold excess of unlabeled IgG1 was subtracted from total binding, and was always <15% of the total.

Endocytosis of ¹²⁵I-IgG1. Endocytosis was measured essentially as described by Jones et al. (17), using nonspecific protease and acid stripping to distinguish internal from total cell-associated radioactivity. Cos cells were transfected with the various constructs as described above. On the second day after transfection, the cells were preincubated for 1–2 h in IM at 37°C. The cells were then washed thrice with chilled PBS and incubated 5 min at 37°C in PBS with 5 mM EDTA. Cells were detached with gentle pipetting and centrifuged 5 min at 300 g. Cells were resuspended in chilled IM at 10⁷/ml, and ¹²⁵I-IgG1 (prepared as described above) was added to a final concentration of 20 nM. Cells were incubated with label for 18 h at 4°C. Cells were centrifuged at 5 g for 3 min and resuspended in fresh chilled IM. Samples were incubated for 20 min; those for binding were at 4°C, while those for endocytosis were at 37°C. All subsequent manipulations were at 4°C. All samples were then centrifuged at 5 g for 3 min. Samples for stripping were resuspended in PBS, 100 U/ml DNase I (Sigma Chemical Co.), and 5 mg/ml Pronase E (Sigma Chemical Co.). Controls were resuspended in PBS, 100 U/ml DNase I, 0.1% OVA (PDO). The stripping incubation was for 1 h. Subsequently, all samples were washed thrice in PDO. Finally, samples for stripping were incubated in 25 mM acetic acid, 0.3 M NaCl (while controls were incubated in PDO) for 3 min. The reaction was terminated by centrifugation through oil (dibutyl phthalate/dioctyl phthalate; 3:2 [vol/vol]). Cell pellets were separated from the oil and were counted by gamma scintillation. All samples were done in duplicate or triplicate, and the experiment reported here was repeated with similar results. Nonspecific binding determined using a 100-fold excess of unlabeled IgG1 was subtracted from total binding, and was always <10% of the total.

Results and Discussion

Fc γ RI-transfected cells have been reported to bind but not ingest EA (7). However, careful analysis revealed very low levels of phagocytosis (Figs. 1 and 2). This low level was clearly distinguishable from the complete absence of phagocytosis seen in mock-transfected or untransfected Cos cells. Examination of electron micrographs of 40 Fc γ RI-transfected cells revealed three cells that had internalized one EA each. The predominant pattern seen in the electron micrographs was one of simple binding to the cell surface with pseudopodia seen very rarely (Fig. 3, A and B).

Our primary goal was to assess whether we could rescue

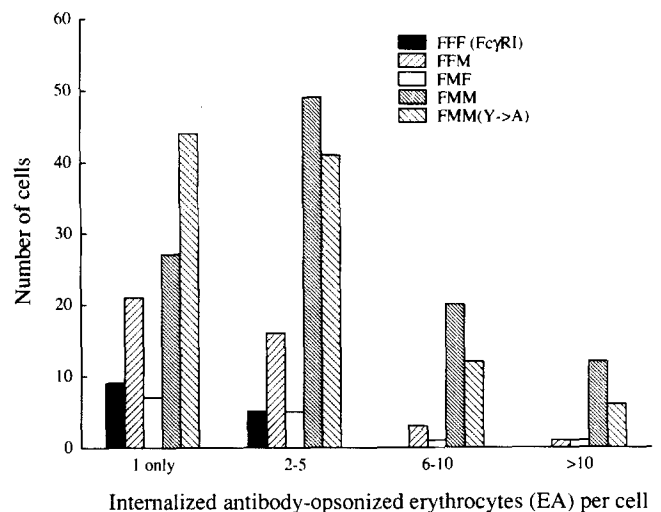


Figure 1. Phagocytosis of EA by Cos cells transiently transfected with DNA encoding the high-affinity Fc γ receptor (FFF) (CD64) and chimeric receptors. Data are presented as the number of cells having the specified number of internalized EA among the population of cells having any internalized EA.

the phagocytic phenotype of this class of Fc receptor in Cos cells by replacing the transmembrane region and cytoplasmic tail with the analogous regions from the macrophage MR. A feature of this experimental system is that we are able to use opsonized erythrocytes as ligands. Erythrocytes may be viewed as passive particles, thereby avoiding the complication that the ligand may contribute to cell entry, as is the case with many microorganisms. In addition, extracellular EA can be effectively removed by hypotonic lysis. Results shown in Fig. 3, C and D, confirm that chimeric receptors that contain the MR transmembrane region and cytoplasmic tail (FMM) mediate phagocytosis that appears morphologically similar to that observed previously in MR-transfected Cos cells (4) and in macrophages. Quantitative analysis of phagocytosis revealed that the phagocytic index of FMM-

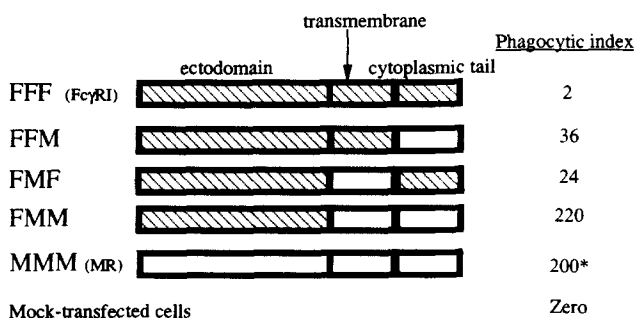


Figure 2. Phagocytosis by chimeric and mutant receptors. Phagocytic index is the number of internal EA per 100 Cos cells, corrected for the fraction of Cos cells expressing the receptor. *The chimeric constructs and the native MR cannot be directly compared, since they recognize different ligands. The phagocytic index quoted for the MR was obtained in a different experiment, using fluorescently labeled zymosan as the ligand, and removing extracellular particles with a brief trypsin treatment.

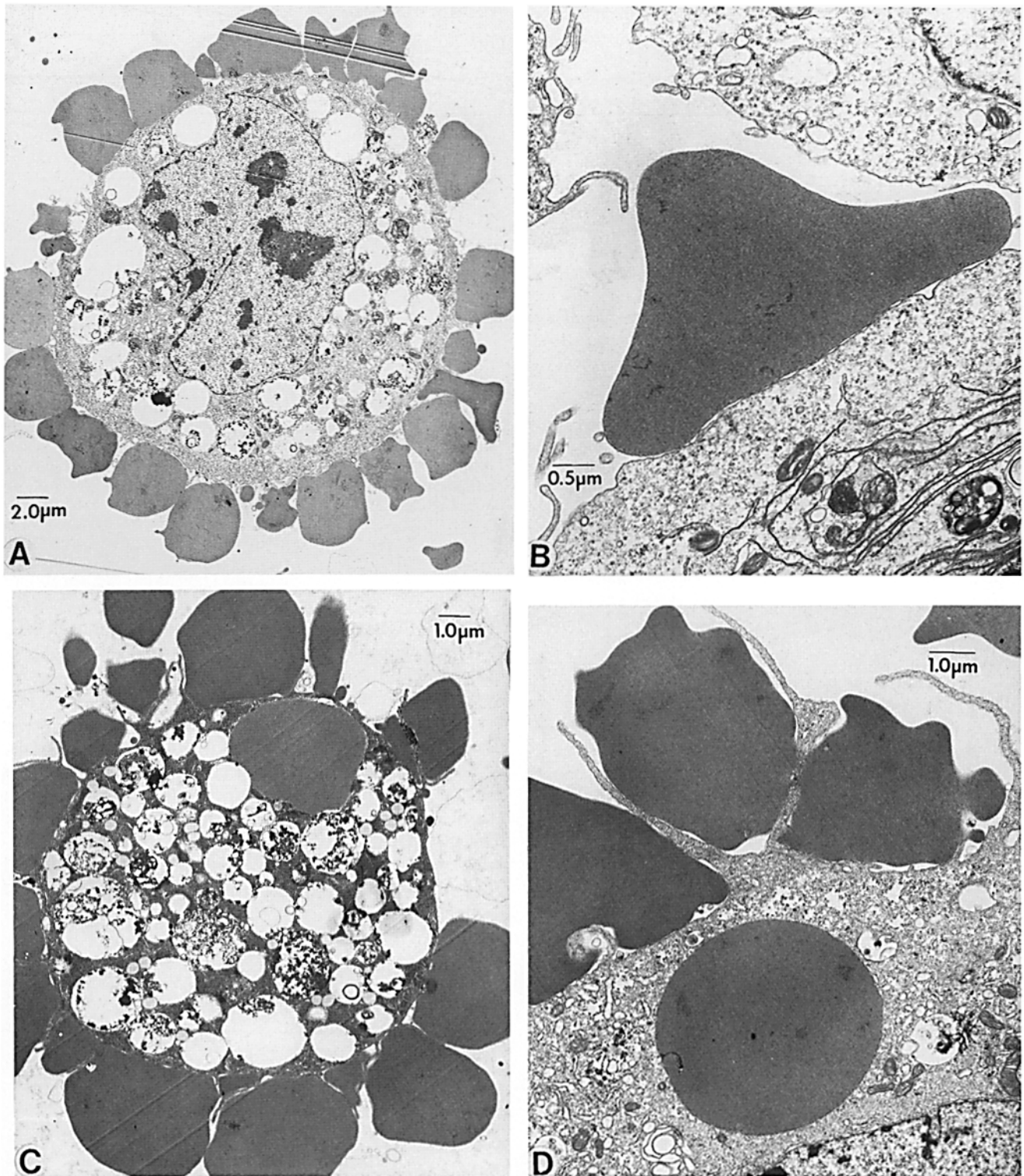


Figure 3. Electron micrographs of transfected Cos cells incubated with opsonized erythrocytes. (*A* and *B*) FFF (Fc γ RI)-transfected cells; (*C* and *D*) FMM-transfected cells. Note internalized EA and pseudopods reaching out to envelope a bound erythrocyte in the FMM-transfected cells, whereas the bound RBC remains inert on the surface of the FFF-transfected cells.

transfected cells was 220, and the distribution of the number of EA per cell ingesting revealed that the majority of cells had two or more internalized EA. A large subset of cells ingested more than five particles (Figs. 1, 2, and 4). These ex-

periments indicated that the MR transmembrane and cytoplasmic domains were sufficient to confer gain of phagocytic function on the Fc γ RI ectodomain in Cos cells. Our next goal was to assess the importance of each of these domains

individually. Interestingly, replacement of either domain alone with the corresponding portion of the MR resulted in suboptimal rescue of the phagocytic phenotype. The phagocytic indices of FFM and FMF were 36 and 24, respectively, compared with two for FcγRI-transfected cells and zero for mock-transfected Cos cells. The majority of Cos cells expressing chimeric receptor with a single MR domain ingested fewer than two particles (Fig. 1). Phagocytosis but not binding of EA was cytochalasin B sensitive (data not shown).

Endocytosis of monomeric IgG1 by the chimeric receptors was assessed to determine whether the different MR domains contributed in a similar fashion to uptake of individual protein molecules. Table 1 demonstrates that, as with phagocytosis, FMM endocytoses efficiently, removing 91% of bound ligand from the cell surface during a 20-min incubation. FFM and FMF were considerably less efficient, suggesting that for endocytosis, as well as phagocytosis, both the transmembrane domain and the cytoplasmic tail are necessary for optimal uptake of ligand. FcγRI also develops a small nonstrippable fraction. In monocytes, FcγRI does not endocytose monomeric IgG (17). Thus, the small nonstrippable fraction seen here in transfected Cos cells could represent a recycling receptor pool or a protease-resistant extracellular compartment, or may represent low-level true endocytosis occurring in this heterologous cell type.

Site directed mutagenesis of the single cytoplasmic tyrosine residue of the MR was performed in the chimera FMM. The tyrosine (in the FENTLY motif) was mutated to an alanine. The mutant chimera was still able to phagocytose, albeit at slightly less than half the phagocytic index of FMM with wild-type MR tail (Fig. 1). Endocytosis of monomeric IgG1 by the Tyr→Ala mutant was similarly reduced to about half that of the wild-type FMM (Table 1).

Table 1. Endocytosis by Chimeric and Mutant Receptors

Construct	Incubation temperature	Fraction nonstrippable
	°C	
FcγRI	4	0.00
	37	0.17
FMM	4	0.11
	37	0.91
FFM	4	0.14
	37	0.39
FMF	4	0.05
	37	0.17
FMM (Tyr→Ala)	4	0.07
	37	0.51

“Fraction nonstrippable” is the proportion of bound radioactivity not stripped by protease and acid treatment, i.e., internalized or otherwise resistant to removal by this treatment. Total cell-associated radioactivity ranged from 6000 to 200,000 cpm based on varying transfection efficiencies for the different constructs. Nonspecific binding was always <10% of total binding.

To determine that the changes in phagocytic and endocytic ability of the chimeric and mutant receptors result from intrinsic changes and are not a function of altered affinity for ligand, we measured the affinity of the various construct receptors for ¹²⁵I-IgG1. After transfection and expression in Cos cells, ¹²⁵I-IgG1 binding was measured at 4°C in the presence of various concentrations of unlabeled IgG1. Half-maximal inhibition of ¹²⁵I-IgG1 binding occurred at ~20

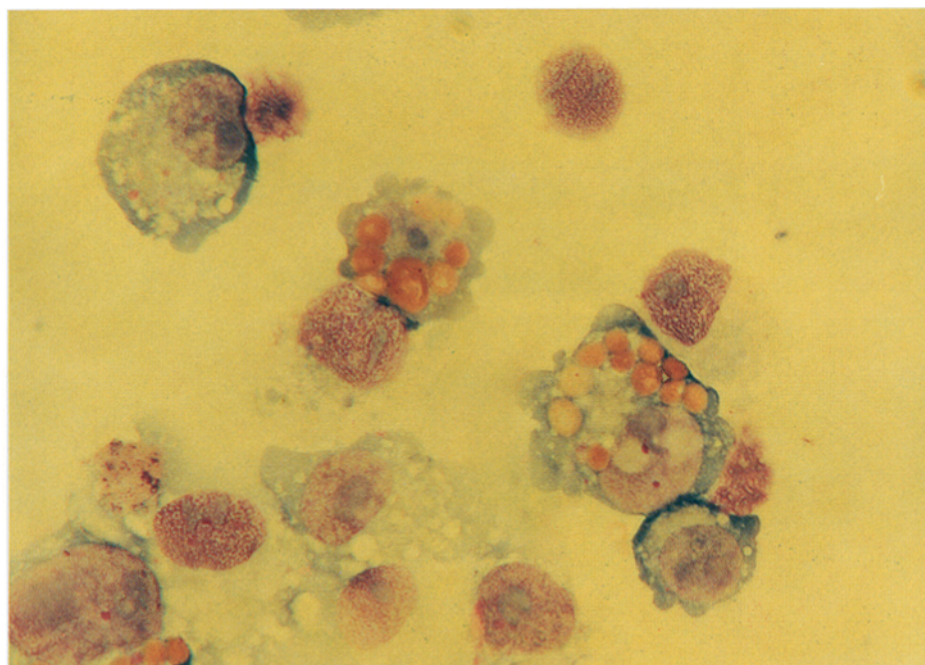


Figure 4. Cytocentrifuge preparation from FMM-transfected Cos cells. Note the large number of internalized EA. Bar, 5 μm.

nM cold IgG1 (identical to the published K_d for Fc γ RI [18]), and was not significantly different in cells transfected with Fc γ RI or the various constructs (Fig. 5 A). In addition, affinity for ligand-coated particles is qualitatively similar in all the constructs. Rosetting of EA by transfected cells shows similar levels with Fc γ RI and each of the chimeric constructs (Fig. 5 B) (FMM Tyr \rightarrow Ala; data not shown).

In this report, we have shown that a phagocytic signal resides in the transmembrane and cytoplasmic domains of the

MR. The presence of information encoding phagocytic competence in the cytoplasmic tail of the MR was to be expected. However, the finding that the transmembrane region contributes in nearly equal measure and that the tail and transmembrane regions are synergistic is novel. A limited number of studies have suggested functional specificity residing in transmembrane segments of some membrane-bound receptors, whereas several other receptors appear to be insensitive to fairly dramatic changes in the transmembrane domain (19–21).

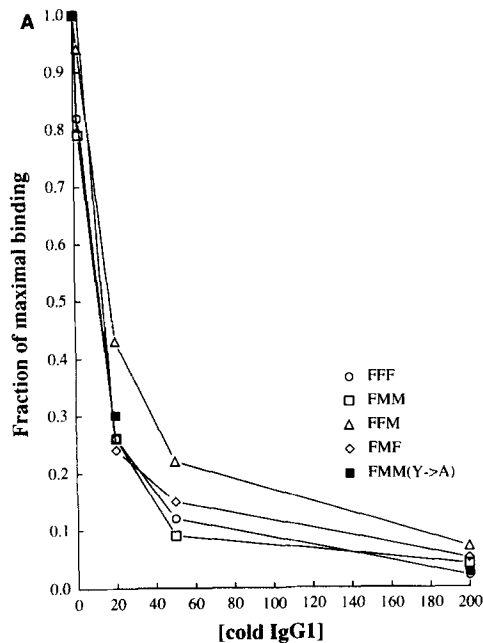
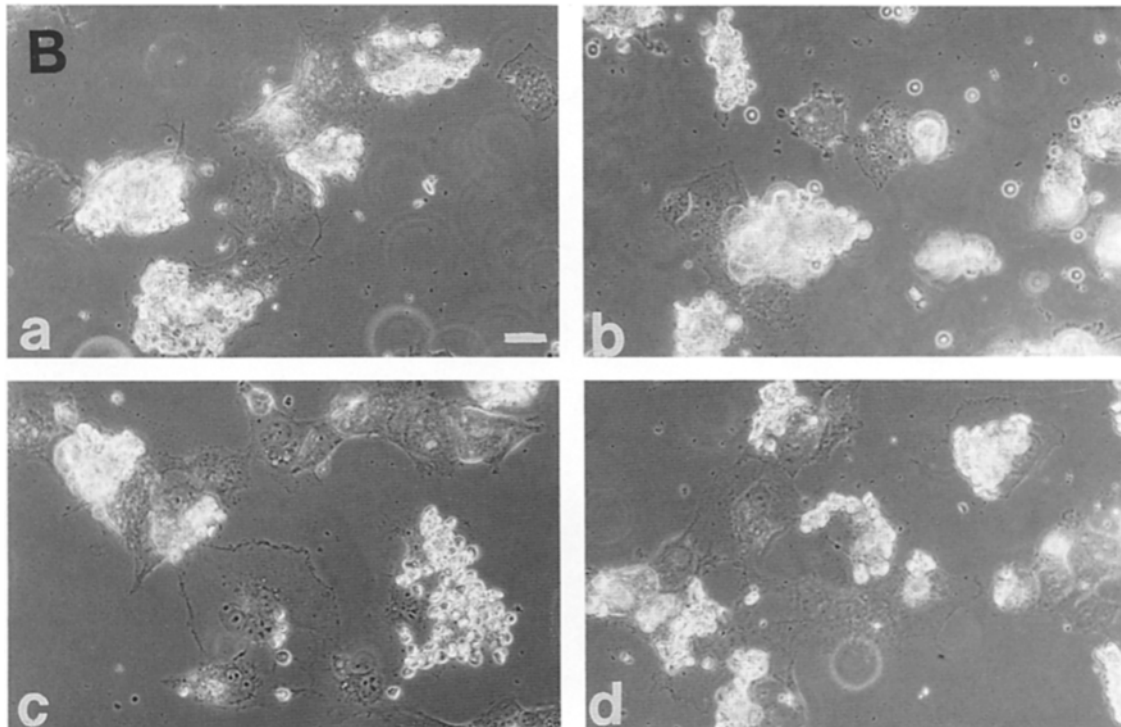


Figure 5. (A) Measurement of affinity for monomeric IgG1 of chimeric receptors expressed in Cos cells by competition of a fixed concentration of 125 I-IgG1 with various concentrations of unlabeled IgG1. (B) Low-power phase-contrast micrographs of Cos cells transfected with each of the constructs and allowed to bind EA. Note the similar number of EA per cell binding EA in each construct, as well as the similar proportion of cells binding EA. a, FFF (Fc γ RI); b, FMF; c, FFM; d, FMM. Bar, 40 μ m.



Transforming point mutations of the *neu* proto-oncogene occurring in the transmembrane region result in clear changes in the tertiary structure of the protein that correlate well with oncogenic activity (22). The transmembrane region of the gp55 envelope glycoprotein of the Friend spleen focus-forming virus has been shown to specify disease phenotype (anemia vs. polycythemia) in chimeric gp55 proteins made from anemia and polycythemia strains, expressed in intact virus (23). Point mutations in the transmembrane domain of membrane-bound IgM can abolish signalling and antigen presentation in a murine B cell line (24).

The mechanism by which the MR transmembrane region plays such a major role in phagocytosis is not clear. Decreased phagocytosis in the chimeras with the Fc γ RI transmembrane domain may be due to the absence of an appropriate phagocytic signal (present in the MR transmembrane domain) or the presence of an antiphagocytic signal in the Fc γ RI transmembrane domain (which might normally be masked by interaction with accessory proteins in the professional phagocyte). The cytoplasmic portion of the MR would be expected to interact with the cellular phagocytic machinery. The transmembrane region might affect the conformation of the cytoplasmic tail, making it active or inactive for phagocytosis. Alternatively, the downstream phagocytic machinery might interact directly with the transmembrane domain via an intra- or transmembrane protein. Some portion of the hydrophobic putative transmembrane region might also be directly accessible to cytosolic machinery. Another manner by which transmembrane domains might affect function has been proposed by Munro (25), who showed that retention of a sialyltransferase within the Golgi apparatus was dependent upon the transmembrane segment and augmented by juxtamembrane extra- and intracellular sequences. In this system, the length of the transmembrane domain seems to be the determinant of function, presumably by presenting the juxtamembrane sequences at the correct spacing. Replacement of the sialyltransferase transmembrane domain by poly-leucine of the correct length resulted in Golgi retention, whereas incorrect lengths allowed translocation to the cell surface (25). Length seems unlikely to be the crucial factor in our case, as the MR and Fc γ RI transmembrane regions differ in length by only a single residue.

Fc γ RI, the high-affinity Fc receptor for IgG, by our analysis and that of others is poorly endocytic and phagocytic when expressed in heterologous cells. Even when expressed in its usual physiological context, it has been reported to dem-

onstrate only nominal endocytosis (17). These results raise the question as to the true function of this molecule, which may serve to focus opsonized ligands that are then internalized by other classes of phagocytically competent Fc receptors. In this manner, it may have a similar function to the first complement receptor, CR1.

The cytoplasmic tail of the MR has only one motif that is homologous to other known receptor tails. The ¹⁴⁰⁶FENTLY¹⁴¹¹ motif resembles a consensus sequence for receptor-mediated endocytosis by the low-density lipoprotein (LDL) receptor (10). Structural analysis of small peptides modeling the LDL receptor's (and another endocytosed protein's) endocytic motif suggests that formation of a tight turn correlates with endocytosis (11, 12). Replacement of tyrosine with alanine disrupts the tight-turn in the peptides, and reduces endocytosis in mutant receptors (10, 11). Other aromatic residues outside of a consensus motif play a role in endocytosis by other receptors (13, 14). We have found that the mutation Tyr¹⁴¹¹→A1a does not abolish phagocytosis or endocytosis by the Fc-MR chimeras, demonstrating that this residue is not essential for either process. However, this mutation reduces both endocytosis and phagocytosis by a similar degree, suggesting that Tyr¹⁴¹¹ may be playing a role in both processes. These results raise the question of the possible role in phagocytosis of clathrin and associated proteins (cf. 26), which are clearly important in receptor-mediated endocytosis.

Efficient phagocytosis has long been thought of as a specialized function of cells of the myelomonocytic lineage, presumed to require coordinated expression of a host of cell-specific gene products. Fc γ RIII, a class of low-affinity Fc receptors expressed in phagocytes, have associated accessory chains that are expressed in hematopoietic cells and that appear to play a critical role in receptor expression (27). However, the Fc γ RII receptors do not appear to share this dependence on accessory proteins, and have recently been shown to mediate phagocytosis when transfected into heterologous cells (7). These findings, our previous data (4), and the studies reported here indicate that phagocytosis can occur in heterologous cells if the appropriate phagocytic receptor is expressed on the cell surface. The chimeric gain of function receptors described here provide a unique system in which critical phagocytic motifs in the MR transmembrane and cytoplasmic regions can be defined. The relationship of putative phagocytic motifs to those critical for receptor-mediated endocytosis can now also be studied.

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