CD47, a Ligand for the Macrophage Fusion Receptor, Participates in Macrophage Multinucleation*

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The macrophage fusion receptor (MFR), also called P84/BIT/SIRPα/SHPS-1, is a transmembrane glycoprotein that belongs to the superfamily of immunoglobulins. Previously, we showed that MFR expression is highly induced at the onset of fusion in macrophages, and that MFR appears to play a role in macrophage-macrophage adhesion/fusion leading to multinucleation. The recent finding that IAP/CD47 acts as a ligand for MFR led us to hypothesize that it interacts with CD47 at the onset of cell-cell fusion. CD47 is a transmembrane glycoprotein, which, like MFR, belongs to the superfamily of immunoglobulins. We show that macrophages express the hemopoietic form of CD47, the expression of which is induced at the onset of fusion, but to a lower level than MFR. A glutathione S-transferase CD47 fusion protein engineered to contain the extracellular domain of CD47, binds macrophages, associates with MFR, and prevents multinucleation. CD47 and MFR associate via their amino-terminal immunoglobulin variable domain. Of the nine monoclonal antibodies raised against the extracellular domain of CD47, three block fusion, as well as MFR-CD47 interaction, whereas the others have no effect. Together, these data suggest that CD47 is involved in macrophage multinucleation by virtue of interacting with MFR during adhesion/fusion.

Osteoclasts and giant cells are characterized by multinucleation and a powerful ability to resorb the substrate onto which they adhere. Although osteoclasts and giant cells play an important role in bone remodeling and immune defense, respectively, they are also associated with osteoporosis, granulomatous diseases, and tumors.

Multinucleation appears to endow macrophages with the capacity to digest and resorb extracellular infectious agents, foreign material, and other components that are too large to be internalized, such as bone. This resorption occurs in an "extracellular lysosomal compartment" sealed off between the multinucleated cell and its target substrate (reviewed in Ref. 1). The plasma membrane that faces the extracellular domain is highly ruffled and specialized. Multinucleation gives macrophages added resorptive capacity, in part by making available a large excess of plasma membrane.

Understanding the mechanism by which macrophages differentiate into osteoclasts and multinucleated giant cells is of extreme importance. One of the key steps in the differentiation of osteoclasts and giant cells is the fusion mechanism of their mononucleated precursor cells. It is assumed that both osteoclasts and giant cells originate from the fusion of mononuclear phagocytes. Despite the pathophysiological importance of these cells, the mechanism by which their mononucleated precursors fuse remains poorly understood. Indeed, cell-cell fusion itself, whether it concerns that of sperm cells with oocytes in fertilization or myoblasts with myoblasts in muscle development, has not been investigated thoroughly. It is proposed that cell-cell fusion involves a set of proteins similar to those used by viruses to fuse with host cells before injecting their DNA or RNA (2). It has been hypothesized that viruses have usurped the fusion protein machinery from their target cells. It is now well accepted that virus-cell fusion requires both an attachment mechanism and a fusogenic peptide. One such example is human immunodeficiency virus attachment, where gp120 binds CD4 on T lymphocytes and macrophages (3, 4), whereas the fusion molecule gp40 triggers the actual fusion event. Although putative fusion molecules mediating sperm-oocyte and myoblast fusion have been reported (5–8), the actual protein machinery governing the attachment and fusion of these cells remains unknown.

To investigate the mechanism of homotypic mononuclear phagocyte fusion leading to the differentiation of osteoclasts and giant cells, our hypothesis has been that macrophage-macrophage fusion, similar to virus-cell fusion, depends on the expression of specific cell surface proteins. To identify such proteins, we had established an in vitro macrophage fusion assay as a model system. When cultured under fusogenic conditions, rat alveolar macrophages rapidly generate large polykaryons whose non-adherent plasma membrane is enriched in sodium pumps while the opposite plasma membrane facing the substrate is enriched in proton pumps (9, 10). This is a property that is shared with osteoclasts (reviewed in Ref. 1). Using these fusing macrophages as immunogen, we previously generated four monoclonal antibodies (mAbs)1 that block fusion.

1 The abbreviations used are: mAb, monoclonal antibody; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; MHC, major histocompatibility complex; PCR, polymerase chain reaction; nt, nucleotide(s); GST, glutathione S-transferase; ELISA, enzyme-linked

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sion. All four mAbs recognize the same antigen, the macrophage fusion receptor (MFR), which is highly and transiently induced at the onset of fusion (11, 12). MFR was cloned simultaneously by several groups as P84/BIT/SIRPα/SHPS-1 (13–15). MFR is a type I transmembrane glycoprotein that belongs to the superfAMILY OF IMMUNOGLOBULINS (Ig) (12). MFR contains three Ig domains in its extracellular part and closely resembles CD47. The intracellular domain of MFR associates with the phosphatases SHP-1 and SHP-2, hence its name, SHPS-1 (15). We reported that the recombinant extracellular domain of MFR engineered as a GST fusion protein blocks fusion by specifically binding to fusing macrophages (12), suggesting that MFR interacts with a putative ligand expressed on the surface of fusing macrophages. Jiang et al. (16) recently reported that IAP/CD47 is a ligand for P84/BIT/SIRPα (17), demonstrating that CD47 is a counterreceptor for human motoneurite outgrowth (17), suggesting that CD47 might be a ligand for the integrin αvβ3 from human placenta (19) prior to being shown to be CD47 (20, 21). CD47 comprises an extracellular immunoglobulin variable domain and five transmembrane domains with its COOH-terminal domain located intracellularly. Its intracellular COOH-terminal domain exists in four alternatively spliced forms (22).

We reasoned that if macrophages express CD47, this molecule might interact with MFR and participate in macrophage adhesion/fusion leading to multinucleation. We present evidence that: (i) fusing macrophages express the hemopoietic form of CD47, (ii) a fusion protein engineered to contain the extracellular domain of CD47 blocks fusion, (iii) three out of nine mAbs directed against the extracellular domain of CD47 block both MFR-CD47 interaction and fusion, and (iv) CD47 and MFR interact via their immunoglobulin variable domain in fusing macrophages. Together, these data suggest that CD47 belongs to the protein machinery that mediates macrophage adhesion/fusion leading to multinucleation.

**EXPERIMENTAL PROCEDURES**

**Cells**—Rat alveolar macrophages were obtained from 12-week-old Fisher 344 rats (Charles River, Kingston, NY) by tracheobronchial lavage and cultured in fusogenic conditions as described previously (9, 10). In brief, cells were plated at a density of 5 × 10^5 cells/ml in MEME supplemented with 10% human serum, and then, once adherent, cultured in 5% human serum.

**Antibodies**—Mouse anti-CD47 monoclonal antibodies were generated by immunizing IAP-deficient mice with purified human placental CD47 (19), fusing spleen cells with the non-secreting myeloma P3 × 63Ag8.653 (ATCC), and screening clones for reactivity with human and mouse CD47.

**Total RNA isolation**—Total RNA was isolated from rat alveolar macrophages immunosorbent assay; PBS, phosphate-buffered saline; MFR, macrophage fusion receptor; MEME, minimum essential medium with Earl’s salts; DAPI, 4’,6-diamidino-2-phenylindole; IAP, integrin-associated protein/CD47; miap, mouse monoclonal antibody anti-IAP/CD47.

**Immunofluorescence**—Cells were cultured in Lab-Tek (Nalge Nunc, Naperville, IL) glass chamber slides for the indicated time in MEME supplemented with 5% human serum and 5% FBS in a 37 °C humid environment at room temperature, and washed for 60 min in PBS-milk (PBS with 5% nonfat dry milk). The cells were incubated for 2 h at room temperature in PBS-milk supplemented with mouse anti-CD47 mAbs (2 μg/ml), mAb 10C4 (cell culture supernatant, 1:100), or control mAb IgG2a (2 μg/ml). Following four washes of 15 min each in PBS-milk, the cells were incubated for an additional 1 h with lissamine-rhodamine-conjugated F(ab’2) donkey anti-mouse IgG (1:100 dilution) in the same buffer. The cells were washed in PBS-glycerol (1:1 v/v) supplemented with 0.5 μg/ml DAPI (Sigma). The images were imaged at 570 and 350 nm using the lissamine rhodamine sulfonyl chloride and DAPI excitation filters, respectively, on an Olympus microscope equipped with UV light.

**RNA isolation, cDNA Synthesis, Polymerase Chain Reaction (PCR), and Cloning**—Total RNA was isolated from rat alveolar macrophages cultured in fusogenic conditions for the indicated times. RNA was extracted using a modification of the methods described by Glisin et al. (23) and Ulrich et al. (24) or the RNaseasy kit (Qiagen, Inc., Chatsworth, CA). In each case guanidinium thiocyanate homogenization buffer was added to the freshly isolated and cultured cells after rapid removal of culture medium. The pellets were resuspended in a lysis buffer with a 20-gauge needle (Beckton Dickinson, Franklin Lakes, NJ). For purification by cesium chloride, 2.5-ml aliquots of the lysate were layered onto a 2-ml cushion of 5.7M cesium chloride (American Bioanalytical, Natik, NJ) in RNase-free 5.1-ml polyallomer centrifuge tubes, which were centrifuged at 150,000 × g for 20 h using a Ti 55 SW rotor. The supernatants were aspirated and the pellets dissolved in TE (pH 7.6) containing 0.1% SDS by freezing and thawing the samples twice and then warming to 45 °C. RNA was precipitated by the addition of 0.3 μl sodium acetate and three volumes of ethanol. The pellets were resuspended in diethylpyrocarbonate-treated water and the concentration determined using optical density measurements taken in a PerkinElmer Life Sciences UV-Visible spectrophotometer (Foster City, CA). First strand cDNA was synthesized as follows: 1 μg of total RNA was reverse transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (Roche Molecular Biochemicals) in a 20-μl reaction primed with oligo(dT)12,18 primer (Promega, Springfield, NJ). The protocol followed was that published in the instruction manual from CLONTECH (Palo Alto, CA). The cDNA was aliquoted and stored at −70 °C. For PCR reaction, 1 μl of cDNA was used as template in a 25-μl PCR mixture containing 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer Life Sciences), 1.25 mM MgCl2, and 0.1 mM dNTP mix (New England Biolabs, Beverly, MA). The buffer condition used for the PCR was a 1-0M dilution of the 10-fold mix provided with the polymerase. The sequences of the primer pairs (used at a concentration of 0.1 μg/reaction) were as follows: full-length CD47, primer forward (nt 24–238) and reverse primer (nt 978–986, 5’TGCTCAAGAACCTGTTATCC-3’). The cycle parameters were 3 min at 95 °C, 1 min at 50 °C, and 3 min at 72 °C for 30 cycles. The PCR fragment was gel-purified using Geneclean (Bio 101 Inc., Branford, CT) and cloned into PCR II TA cloning vector (Invitrogen, San Diego, CA) using AmpliTaq DNA polymerase and fluorescent dyeoxy terminators (Perkin Elmer Life Sciences) in a cycle sequencing method. The resulting DNA fragments were gel-purified and analyzed using an automated Applied Biosystems 373A stretch or 377 DNA sequencer.

**MFR, CD47, and MHCII ELISA**—MFR, CD47, and MHCII cell surface expression was quantified by ELISA as follows; 5 × 10^4 alveolar macrophages/well plated at 5 × 10^5 cells/ml in 96-well plates were cultured for the indicated times. The minimum culture time after plating in each experiment was 1 h in order to secure the adherence of the cells to the wells. The cells were fixed at room temperature in 4% paraformaldehyde for 10 min, and treated with 3% hydrogen peroxide for 5 min at room temperature. The cells were then incubated in 100 μl of PBS supplemented with 5% dry milk (PBS-milk) for 2 h. The cells were subsequently incubated overnight with mAb 10C4 (cell culture supernatant), mAb anti-CD47 (2 μg/ml), or mAb anti-MHCII (10–50 μg/ml). Following three washes of 10 min each with PBS, the cells were incubated at room temperature for 2 h in PBS-milk supplemented with goat anti-mouse IgG-horseradish peroxidase conjugate (1:5000 dilution). The cells were washed three times with PBS for 10 min each. Antibody binding was quantitated by incubating the cells for five min in 100 μl of 3,3’,5,5’-tetramethylbenzidine (HRP substrate, matrix, and physical properties of the material being analyzed. The results should be expressed in a clear, concise, and logical manner, ensuring that they are relevant and support the conclusions drawn. The structure of the text ensures that the reader can follow the reasoning and the flow of ideas throughout the document. The use of figures and tables, if present, should be justified and clearly explained in the text. The overall organization of the paper is logical and coherent, with a clear introduction, methods, results, and discussion sections. The results are presented in a structured format, making it easy to follow the flow of the data and conclusions. The conclusions are directly linked to the results and are supported by the data presented. The paper is well-written, with proper citation of sources and adherence to the formatting guidelines.
Moss Inc., Pasadena, MD). Optical density (OD405) measurements were made using a kinetic reader (Menlo Park, CA).

Immunoprecipitation, Pull-down, and SDS-PAGE Analysis—Rat alveolar macrophages were harvested by lavage, plated in six-well plates at 5 × 10⁶ cells/ml, cultured in MEME supplemented with 5% HS for 2 days (immunofluorescence) or 4 days (immunoprecipitation), and to pull-down as follows. In brief, cells were lysed in phosphate-buffered saline supplemented with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, a mixture of protease and phosphatase inhibitors (aprotinin, leupeptin, and pepstatin, each at 10 μg/ml; 0.1 mM phenylmethylsulfonyl fluoride, one tablet of Complete™ (Roche Molecular Biochemicals), 0.1 mM sodium orthovanadate, and sodium fluoride. For immunoprecipitation, the post-nuclear supernatants were pre-incubated with mouse IgG1 or non-immune rabbit serum for 1 h, then with protein G-agarose (Upstate Biotechnology) (1 μg/ml) for 30 min. The lysates were then incubated with miaps or rabbit anti-MFR for 1 h, followed by protein G-agarose for 30 min. For “pull-down” experiments, the post-nuclear supernatants were pre-incubated with GST that was coupled to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 30 min and then incubated with GST-C47e or GST-MFRev coupled to glutathione-Sepharose 4B beads, for 2 h. The immunoprecipitates and the pull-downs were analyzed by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions followed by Western blot analysis using mAb 10C4 and miap 460-biotin, then HRP-rat anti-mouse F(ab’)2 and HRP-ABC, respectively.

Western Blot Analysis—Alveolar macrophage lysates, immunoprecipitates, and pull-downs were subjected to electrophoresis on a 10% polyacrylamide gel in reducing or non-reducing conditions. The proteins were transferred onto nitrocellulose membranes (Millipore Corp., Bedford, MA) by electroblotting for 90 min at 4 °C. The membranes were subsequently incubated in PBS supplemented with 5% dry milk overnight at 4 °C, washed in PBS supplemented with Tween 20 (1%), and incubated with mAb 10C4, miap 460-biotin, anti-MFR, or anti-SHP-1 for 1 h at room temperature, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse, HRP-conjugated donkey anti-rabbit Ig, or HRP-ABC, accordingly, for 30 min at room temperature. The HRP activity was determined using ECL (Amersham ECL kit). The enzyme reaction was performed according to the manufacturer’s instructions and the blots exposed to x-ray films.

Production of GST-MFRev: Recombinant Soluble Extracellular V1 Domain of MFR (MFRev) Fused to GST—MFRev was expressed as a GST fusion protein using PGEX-4T-1 (Amersham Pharmacia Biotech). PCR amplification of the extracellular domain of MFR was performed using a primer that lacked the two extracellular C1 loops, as the template. The resulting PCR product was extensive dialyzed against PBS. Recombinant GST-MFRev ran approximately 40 kDa and was used to transform the protease-deficient Escherichia coli strain BL-21. Soluble GST-C47e was isolated from 2 liters of bacterial culture using the bulk GST purification module as described by the manufacturer’s recommendations. The eluted protein was extensively dialyzed against PBS and stored at −70 °C until ready for use. Recombinant GST-C47e was quantified in the same way as GST-MFRev.

Fusion Assay Using GST-C47e and GST-MFRev—Fusion proteins were stored in lipopolysaccharide-free water as 1 mg/ml stock solutions, diluted in MEME, and filter-sterilized before use. Aliquots (10 μl) of 5 × 10⁶ alveolar macrophages/ml were plated in quadruplicate using 96-well tissue culture plates, cultured in MEME with 5% human serum supplemented or not with recombinant proteins (GST-C47e, GST-MFRev, and GST) (1–50 nM). The cells were examined daily for 4 days, and fusion was graded blindly by three investigators on a scale of 1 (absence of fusion) to 5 (fusion greater than 90%), as described previously (12).

Binding Assays Using Recombinant GST-C47e and GST-MFRev Fusion Proteins—Freshly isolated rat alveolar macrophages were plated at 5 × 10⁶ cells/ml in quadruplicate using 96-well plates (10⁶ cells/well). The cells were cultured for 24 h in MEME plus 5% human serum, fixed in 4% paraformaldehyde for 15 min at room temperature, and blocked with PBS plus 5% milk. The cells were supplemented with GST-C47e at the indicated concentrations. Binding proceeded overnight at 4 °C. For disassociation studies, the media were replaced with fresh medium lacking recombinant protein to allow for disassociation. Disassociation proceeded at 4 °C for the indicated times. The media from all wells were then removed, and the cells were fixed for 30 min in 4% paraformaldehyde. Following washes and blocking in PBS plus 5% milk, the cells were incubated with mouse anti-GST conjugated to either biotin or HRP (2 μg/ml) for 30 min, followed by HRP-ABC for 30 min at 4 °C for biotin-anti-GST. HRP was reacted by adding 100 μl of peroxidase substrate (TMB, Moss Inc, Pasadena, MD) to each well and the optical density was read at 650 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA). For specific binding studies, cells were incubated with increasing concentrations of GST-C47e or GST overnight at 4 °C. For competition binding studies, cells were preincubated with increasing concentrations of either mAb 10C4 or miaps for 1 h at 4 °C, then supplemented with either GST-C47e or GST-MFRev (20 nm) overnight, respectively. Cells were subsequently reacted as described above to detect GST fusion protein binding.

Statistical Analysis—Data are expressed as mean ± 1 standard deviation. Comparative analyses of the means were performed with appropriate controls using independent Student’s t test to determine the 95% confidence level (p < 0.01).

RESULTS

CD47 Is Expressed by Fusing Macrophages—To investigate whether fusing macrophages express CD47, rat alveolar macrophages were plated in glass chamber slides and cultured under fusogenic conditions. Under these conditions, 90–95% of the cells fuse within 4–5 days into multinucleate giant cells that contain hundreds of nuclei each. This is shown in Fig. 1A, where multinucleated cells were reacted with lissamine-rhodamine-conjugated antibodies (red fluorescence, left panels), as well as with the nuclear stain DAPI (light blue dots, center panels), and visualized with Hoffman modulation contrast (right panels). Fusing cells were subject to indirect immunofluorescence using a panel of nine anti-CD47 mouse mAbs that we generated by immunizing CD47-deficient mice with the extracellular domain of human CD47 (miaps, see “Experimental Procedures”). As shown in Fig. 1A (left panels), intact fusion multinucleated macrophages reacted strongly with both miap 410 and miap 450. In contrast, the signal detected on most of the plasma membrane of multinucleated cells appeared diffuse, and less intense. Of importance, each anti-CD47 mAb miap gave similar results (data not shown), whereas isotype-matched controls gave no signal (Fig. 1A, and data not shown).

The localization of CD47 appeared similar to that of mAb 10C4, which recognizes MFR (Fig. 1A), and concentrated on the plasma membrane of fusing multinucleated macrophages. Once multinucleated, macrophages appeared to express a
lesser amount of CD47. This is a pattern of expression similar to that of MFR (11).

To determine whether the cell surface expression of CD47, like MFR, was altered by multinucleation, fusing macrophages were subjected to ELISA at different time points after plating. As shown previously, the expression of MFR is highly induced at the onset of multinucleation, i.e. 24 h after plating, and decreases thereafter with multinucleation (12). CD47 expression was also induced 1 day after plating, but to a lesser extent than MFR (Fig. 1B), and continued to increase through day 2. Thereafter, like MFR, CD47 expression tended to decrease with multinucleation. These results were confirmed by Western blot analysis of fusing macrophage lysates at various times after plating (Fig. 1C). When CD47 was analyzed under non-reducing conditions, it ran as an 87–91-kDa doublet, probably due to its multimerization. Of importance, mAb 10C4 (anti-MFR) recognized an additional band of 90 kDa that was most obvious 24 h after plating, indicating that it was also induced. Aliquots of the same cell lysates were analyzed in parallel by Western blot under reducing conditions using a polyclonal antibody that

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**Fig. 1. Expression of CD47 by fusing macrophages.**

a, freshly isolated rat alveolar macrophages were plated in Lab-Tek glass chamber slides at a density of $5 \times 10^6$/ml and cultured in MEME supplemented with 5% human serum for 4 days. The cells were fixed with paraformaldehyde and reacted with miap 410, miap 450, mAb 10C4, or IgG2a, followed by lissamine-rhodamine-conjugated Fab', donkey anti-mouse IgG. The cells were mounted in PBS-glycerol (1:1) supplemented with the nuclear stain DAPI, and viewed at 570 and 350 nm using the lissamine rhodamine sulfonyl chloride and DAPI excitation filters, or with Hoffman modulation contrast (original magnification, ×100). Note that the multinucleated macrophages contain hundreds of nuclei (light blue dots), and that fusing mononucleated cells demonstrate a positive fluorescent signal that is similar with miap 410, miap 450, or mAb 10C4. Of importance, unfused or fusing (i.e. near the surface of giant cells) mononucleated cells demonstrate a signal for miap 410, miap 450, or mAb 10C4. b, cells were plated as in a but in 96-well plates, and reacted with mouse mAb 10C4 (anti-MFR), miap 430 (anti-CD47), and anti-MHCII followed by HRP-conjugated goat anti-mouse IgG at the indicated times. Standard deviations are less than 5% and cannot be seen (n = 3). c, cells were plated as in a but in six-well plates, using $0.5 \times 10^6$ cells/well. Cells were collected at the indicated times and subjected to SDS-PAGE in non-reducing (upper panel) and reducing (lower panel) conditions, followed by Western blot analysis using mAb 10C4 and miap 460-biotin (2 µg/ml), followed by HRP-rat Fab' goat (1/5000) and HRP-ABC (1/500), respectively (upper panel); rabbit anti-MFR and anti-SHP-1 followed by HRP-conjugated donkey anti rabbit (lower panel).
recognizes the intracellular domain of MFR. The 90-kDa protein was again detected, at low abundance, but was induced with kinetics similar to that of the 150-kDa MFR (Fig. 1C, lower panel). Although the 90-kDa protein could represent a degradation product of MFR, it may also correspond to the short transcript that lacks exons 3 and 4 encoding the second and third immunoglobulin domains (25, 26). To confirm the even loading of proteins in each lane, the blot was reprobed with an antibody directed against the tyrosine phosphatase SHP-1 whose abundance remained unaffected by multinucleation (Fig. 1C, lower panel). Together, our results show that CD47 expression is transiently induced in fusing macrophages, but to a lower level than both forms of MFR, e.g. the 150-kDa and 90-kDa forms.

Because CD47 is expressed as four different isoforms that differ from each other at their cytoplasmic carboxyl termini (22), we sought to determine which CD47 isoform was expressed by fusing macrophages. Total RNA from fusing macrophages was subjected to reverse transcriptase-PCR using primer pairs designed from the rat CD47 cDNA sequence to generate a full-length CD47 cDNA. The PCR product was then subjected to DNA sequencing. This analysis revealed that the dominant form of CD47 expressed by fusing macrophages is isoform 2, which also predominates in bone marrow cells (data not shown).

Monoclonal Antibodies Anti-CD47 Block Fusion/Multinucleation—The identification of CD47 as a MFR ligand suggested that it might play a role in fusion. To investigate this possibility, we tested whether our anti-CD47 mAbs inhibited macrophage fusion. Among the nine mAbs tested, three blocked fusion in a concentration-dependent manner (miaps 430, 450, and 470) (Fig. 2, A and B; Table I; and data not shown). mAb miap 400 is representative of the 6 miaps that did not alter multinucleation (Fig. 2A, and data not shown). Indeed, this is not unexpected since other anti-human CD47 mAbs can be divided into two groups based on whether or not they inhibit function: in this case, in the regulation of Fc receptor-dependent phagocytosis (19). This suggested that different mAbs miaps recognize distinct sites on the CD47 Ig domain.

Recombinant GST-CD47e Fusion Protein Blocks Fusion/Multinucleation—To further investigate the role of CD47 in macrophage fusion, we engineered a GST fusion protein that contains the NH2-terminal extracellular Ig variable domain of CD47 (GST-CD47e) and tested its ability to bind fusing macrophages. As shown in Fig. 3 (A and B), GST-CD47e bound fusing macrophages in a concentration-dependent, saturable, and reversible manner. When GST-CD47e was added to fusing macrophages, multinucleation was inhibited in a concentration-dependent manner with a maximal effect at 20 nM (Fig. 3C), a potency similar to that of GST-MFRev (12). In contrast, GST did not block fusion. This suggested that the domain of MFR important for macrophage fusion/multinucleation interacts with CD47. Indeed, the inhibition of fusion by anti-CD47 mAbs suggested that CD47 is actively involved in cell-cell interaction. These results are consistent with a role for CD47-MFR interaction in macrophage adhesion/fusion leading to multinucleation.

MFR and CD47 Associate in Fusing Macrophages—To investigate whether CD47 associates with MFR, we took two complementary approaches. First, we subjected lysates from fusing macrophages to immunoprecipitation using anti-CD47 mAb and tested for co-precipitation with MFR by subjecting the precipitate to SDS-PAGE in non-denaturing conditions followed by Western blotting using anti-MFR mAb 10C4. The blot was reprobed with biotinylated anti-CD47 mAb miap 460 to confirm the precipitation of CD47. As a control, MFR was immunoprecipitated with a rabbit polyclonal antiserum that recognizes the intracellular domain of MFR and the immunoprecipitate Western-blotted with biotinylated anti-CD47 mAb miap 460. The blot was reprobed with anti-MFR mAb 10C4. In parallel, macrophage lysates were subjected to immunoprecipitation using mouse IgG1 and nonimmune rabbit serum, used as controls. As a second approach, we subjected lysates from fusing macrophages to pull-down using GST-CD47e and GST-MFRev and tested for co-precipitation with MFR by subjecting the precipitate to SDS-PAGE in non-denaturing conditions followed by Western blotting using anti-MFR mAb 10C4. Among the nine mAbs tested, three blocked fusion: in this case, in the regulation of Fc receptor-dependent phagocytosis (19). This suggested that different mAbs miaps recognize distinct sites on the CD47 Ig domain.

TABLE I

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<tr>
<th>Medium</th>
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<td>Control</td>
<td>5.0 (0.1)</td>
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<tr>
<td>mAbs anti-CD47</td>
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<tr>
<td>miap 430 (ident for miap 450 and 470)</td>
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<tr>
<td>40 µg/ml</td>
<td>1.0 (0.1)</td>
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<tr>
<td>4 µg/ml</td>
<td>1.8 (0.2)</td>
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<tr>
<td>1 µg/ml</td>
<td>5.0 (0.1)</td>
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<tr>
<td>miap 400 (ident for miap 410, 420, 440, 460, and 480)</td>
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<tr>
<td>40 µg/ml</td>
<td>5.0 (0.5)</td>
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<td>GST (20 nM)</td>
<td>5.0 (0.4)</td>
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<td>GST-CD47e</td>
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<td>50 nM</td>
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<tr>
<td>25 nM</td>
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<td>10 nM</td>
<td>3.2 (0.5)</td>
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<td>5 nM</td>
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<tr>
<td>GST-MFRev</td>
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<tr>
<td>50 nM</td>
<td>1.0 (0.1)</td>
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<td>25 nM</td>
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* 5, maximum fusion; 1, absence of fusion (n = 4). Standard deviations are indicated in parentheses.
MFRev (containing the V loop of MFR, see below, and Fig. 4) coupled to glutathione-Sepharose beads. The lysates were first pre-cleared using GST alone coupled to glutathione-Sepharose beads. The material pulled down was analyzed by SDS-PAGE in non-denaturing conditions followed by Western blot as above, using mAb 10C4 and miap 460-biotin, respectively. Together, these experiments revealed that CD47 and MFR associate with each other (Fig. 4, A and B), indicating that MFR interacts with CD47 from fusing rat alveolar macrophages. Of significance, only anti-CD47 mAbs that did not block fusion co-precipitated MFR with CD47 (data not shown). This suggested that mAbs that block fusion do so by preventing CD47-MFR interaction, and that MFR interacts with CD47 during cell-cell adhesion/fusion. Although the mAb anti-CD47 miap 460 precipitated both forms of MFR, GST-MFRev pulled down the 150-kDa form only. This could suggest that the IgV domain of MFR interacts with a domain of CD47 that is not represented in GST-CD47e, or else simply reflect differences in yield since the 90-kDa form of MFR is rather less abundant than the 150-kDa form, hence not detected.

The Immunoglobulin V1 Domain of MFR Is Sufficient to Block Multinucleation—The fact that the 90-kDa form of MFR was recognized by both mAb 10C4 and a polyclonal antibody directed against the intracellular domain of MFR strongly sug-

Fig. 3. GST-CD47e binds fusing alveolar macrophages and blocks fusion. Alveolar macrophages were plated at 5 × 10⁶/ml in six-well plates, cultured in MEME supplemented with 5% human serum for 24 h, fixed with 4% paraformaldehyde, blocked in PBS plus 5% milk, and incubated overnight with GST-CD47e at the indicated concentrations for saturation binding studies (a) and at 20 nM for dissociation binding studies (b). Binding of the fusion proteins was revealed using HRP-conjugated mouse anti-GST. c, cells were cultured as in a, and the medium was supplemented with GST, GST-MFRev, and GST-CD47e at the indicated concentrations. Original magnification, ×100.

Fig. 4. MFR and CD47 associate. a, alveolar macrophages were plated at 5 × 10⁶/ml in six-well plates, 1.2 × 10⁶ cells/well, cultured in MEME supplemented with 5% human serum for 24 h. The cells were lysed and subjected to immunoprecipitation (IP) using anti-CD47 miap 460 and anti-MFR. The lysates were analyzed by SDS-PAGE in non-reducing conditions followed by Western blot (wb) using mAb 10C4 (MFR) and biotin-miap 460 (CD47), b, cells were plated and cultured as in a, but the lysates were subjected to pull-down procedure using GST-CD47e or GST-MFRev coupled to glutathione-Sepharose 4B beads, after pre-clear with GST alone coupled to glutathione-Sepharose 4B beads. The pull-downs were analyzed by SDS-PAGE in non-reducing conditions followed by Western blot (wb) using mAb 10C4 (MFR) and biotin-miap 460 (CD47). Total cell lysates (TCL) were analyzed in parallel. c, GST-MFRev and GST recombinant proteins (1 μg/lane) were analyzed by SDS-PAGE in non-reducing conditions followed by Western blot using mAb 10C4 (MFR) and goat anti-GST.

MFR (containing the V loop of MFR, see below, and Fig. 4C) coupled to glutathione-Sepharose beads. The lysates were first pre-cleared using GST alone coupled to glutathione-Sepharose beads. The material pulled down was analyzed by SDS-PAGE in non-denaturing conditions followed by Western blot as above, using mAb 10C4 and miap 460-biotin, respectively. Together, these experiments revealed that CD47 and MFR associate with each other (Fig. 4, A and B), indicating that MFR interacts with CD47 from fusing rat alveolar macrophages. Of significance, only anti-CD47 mAbs that did not block fusion co-precipitated MFR with CD47 (data not shown). This suggested that mAbs that block fusion do so by preventing CD47-MFR interaction, and that MFR interacts with CD47 during cell-cell adhesion/fusion. Although the mAb anti-CD47 miap 460 precipitated both forms of MFR, GST-MFRev pulled down the 150-kDa form only. This could suggest that the IgV domain of MFR interacts with a domain of CD47 that is not represented in GST-CD47e, or else simply reflect differences in yield since the 90-kDa form of MFR is rather less abundant than the 150-kDa form, hence not detected.

The Immunoglobulin V1 Domain of MFR Is Sufficient to Block Multinucleation—The fact that the 90-kDa form of MFR was recognized by both mAb 10C4 and a polyclonal antibody directed against the intracellular domain of MFR strongly sug-
suggested that the short form of MFR contained only one extracellular Ig linked to the intracellular domain (12, 25–27). The fact that both forms of MFR co-immunoprecipitated with CD47 suggested that it is the single IgV domain present in the short form of MFR that interacts with CD47. To test this possibility, we engineered a GST fusion protein that contained the IgV domain of MFR fused to GST (GST-MFRev). As shown in Fig. 3C, GST-MFRev blocked multinucleation with a potency similar to that of GST-MFRe, i.e. 20 nM (12). mAb 10C4 (anti-MFR) also blocks fusion, and as expected immunoblots GST-MFRev (Fig. 4C), showing that it recognizes the amino-terminal IgV domain of MFR. Together, these data show that the IgV domain of MFR plays an important role in macrophage multinucleation by interacting with CD47.

**MFR and CD47 Interact in Fusing Macrophages—**To investigate whether CD47 interacts with MFR in situ, in intact fusing cells, we performed competitive binding studies between the mAbs and the GST fusion proteins that block fusion in macrophages cultured under fusogenic conditions for 24 h, then fixed. Cells were incubated for 1 h at 4 °C with increasing concentrations of either mAb 10C4 or miap 450, both of which block fusion. Either GST-CD47e or GST-MFRev were then added and detected using HRP-anti-GST antibody. As shown in Fig. 5A, both mAb 10C4 and miap 430 prevented the binding of the recombinant proteins GST-CD47e and GST-MFRev, respectively, in a concentration-dependent manner. This indicated that the antibodies and the ligands bind to the same epitopes, and suggested that they inhibit fusion by preventing MFR-CD47 interaction. Of importance, miaps 430 and 470, which block fusion, gave similar results, whereas miaps that did not block fusion failed to prevent GST-MFRe binding to macrophages (Fig. 5B, and data not shown). Together, these results suggest that MFR and CD47 interact via their variable domain in fusing macrophages.

We then reasoned that if MFR and CD47 interact during fusion, the kinetics of GST-MFRe and GST-CD47e binding sites expression on fusing macrophages should mirror that of their ligand, i.e. CD47 and MFR previously detected by ELISA and Western blot analysis (Fig. 1, B and C). To verify this possibility, macrophages were cultured in fusogenic conditions for increasing amounts of time and subjected to ELISA using GST-MFRe and GST-CD47e as ligands, as described in Fig. 3 (A and B). GST fusion proteins were detected as described above. The data presented in Fig. 5C confirm that the kinetics of GST-MFRe and GST-CD47e binding sites expression is similar to that of CD47 and MFR expression.

**DISCUSSION**

Our hypothesis has been that macrophage fusion, like virus-cell fusion, is mediated by a set of surface proteins that interact in a ligand-receptor manner. The identification of the ligand for MFR was a key step to further our understanding of the fusion mechanism in macrophages. We have now generated evidence that CD47 plays a role in macrophage adhesion/fusion leading to multinucleation by virtue of interacting with MFR. CD47, like MFR, belongs to the superfamily of immunoglobulins. We report here that CD47 and MFR interact with each other, at least in part, via their immunoglobulin variable domain to promote macrophage multinucleation.

An important consideration regarding macrophage fusion is that it involves a homotypic interaction, in contrast to the interaction occurring between neurons at synaptic sites, and between viruses with host cells. This implies that plasma membranes of both cells are endowed with the same set of molecules and interact in a reciprocal manner (Fig. 6). With this reasoning, it is attractive to speculate that the short form of MFR, although poorly expressed, brings the opposite plasma mem-

![Fig. 5. Competitive binding between MFR and CD47.](http://www.jbc.org/)

branes close enough to facilitate fusion by virtue of interacting with CD47 (Fig. 6). This interaction, mediated by the V loops of MFR and CD47, could reduce the distance between two cell

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*Figure 5: Competitive binding between MFR and CD47.*

*a.* alveolar macrophages were plated at 5 × 10⁶/ml in 96-well plates, cultured in MEME supplemented with 5% human serum for 24 h, fixed with 4% paraformaldehyde, blocked in PBS plus 5% milk, and incubated for 1 h with increasing concentrations of mAb 10C4 or miap 430, then overnight with 20 nM GST-CD47e or GST-MFRev, respectively. GST fusion protein binding was detected using HRP-conjugated mouse anti-GST antibody. *b.* same as in *a.*, but macrophages were preincubated with 20 ng/ml miap 410, 450, 460, or 470, then incubated overnight with 20 nM GST-CD47e or GST-MFRev, respectively. GST fusion protein binding was detected using HRP-conjugated mouse anti-GST antibody. *c.* same as in *a.*, but macrophages were cultured as in *a.*, but fixed with paraformaldehyde at the indicated times. Cells were incubated overnight with GST-CD47e or GST-MFRev (20 nM), and fusion protein binding was detected as indicated in *a.*
plasma membranes down to 5–10 nm. Although neither MFR nor CD47 show any homology with known viral fusion proteins, they could potentially facilitate fusion by utilizing one system that combines two functions, attachment and fusion. Accordingly, MFR and CD47 may constitute the “minimal fusion machinery” proposed by Weber et al. (28) for intracellular membrane fusion. Meanwhile, the trans-association between the short form of MFR and CD47 may secure the mononucleated status of macrophages. 

Our results clearly indicate that the surface expression of CD47 is evenly distributed on the surface of mononucleated fusing macrophages, but induced to a lesser extent than MFR. If CD47 is the only ligand for MFR, then the regulation of multinucleation rests mainly upon MFR whose regulation of expression remains to be investigated. The plating of alveolar macrophages at a concentration that allows cell-cell contact is sufficient to induce MFR expression. This induction is transient as multinucleated cells express lesser amount of MFR (11, 12). The question as to why is MFR highly induced, and not so much CD47, at the onset of fusion remains unclear. One possible explanation is that the long form of MFR saturates CD47, thereby preventing its interaction with extracellular ligands, such as thrombospondin (29), and securing a stable cell-cell contact. This may allow relatively low copy numbers of short MFR molecules to closely interact with CD47, and facilitate fusion (Fig. 6). Another possibility is that MFR molecules are capable of homotypic interaction. However, native gel electrophoresis and binding data have failed to reveal evidence of significant homotypic interaction (Fig. 1C and data not shown). Another possibility is that MFR interacts with another ligand, such as CD44, which is also strongly induced at the onset of fusion (30), in which case the regulation of fusion would rely on both MFR and CD44, simultaneously. This last possibility remains to be investigated. We have shown that CD47 and MFR, as well as MFR-CD47 interaction, can regulate the process of macrophage multinucleation. Experiments with CD47-deficient macrophages should reveal whether or not this molecule is essential for the fusion process and at which step it acts.

Vaccinia and variola viruses express proteins which are related to CD47 (31). Although A38L is not known as the actual fusion protein, like CD47, A38L promotes Ca2+ entry into cells possibly by forming a pore (32). Indeed, pore formation is a classical tactic used by parasites to enter host cells (33). Of note, the overexpression of the pore forming P2Z/P2X7, receptor for ATP leads to cell-cell fusion, but is followed by cell death. Likewise, the overexpression of CD47 or A38L leads to cell death (34). This raises the possibility that once the membranes from opposite cells are closely apposed and stable, CD47 molecules may create a pore that triggers cell-cell fusion. Although this last possibility is highly speculative, it opens an interesting avenue of research. Together, our data further support the important role played by MFR in macrophage multinucleation and reveal CD47 as a key partner in this venture. We view MFR and CD47 as potential members of a adhesion/fusion machinery that mediates multinucleation of macrophages. The identification of these molecules will help elucidate the molecular biomechanics of macrophage fusion.

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Addendum—Since this manuscript was submitted for publication, two articles that report on the interaction between MFR and CD47 have been published (35, 36).

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CD47 and Macrophage Multinucleation

CD47, a Ligand for the Macrophage Fusion Receptor, Participates in Macrophage Multinucleation
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