Interaction between the integrin Mac-1 and signal regulatory protein α (SIRPα) mediates fusion in heterologous cells

Marketa Hlavackova, Nataly P. Podolnikova, Yifei Wu, Valentin P. Yakubenko, James Faust, Arnat Balabiyev, Xu Wang, and Tatiana P. Ugarova

From the Center for Metabolic and Vascular Biology, School of Life Sciences, and School of Molecular Sciences, Arizona State University, Tempe, Arizona 85287 and the College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614

Macrophage fusion leading to the formation of multinucleated giant cells is a hallmark of chronic inflammation. Several membrane proteins have been implicated in mediating cell–cell attachment during fusion, but their binding partners remain unknown. Recently, we demonstrated that interleukin-4 (IL-4)-induced fusion of mouse macrophages depends on the integrin macrophage antigen 1 (Mac-1). Surprisingly, the genetic deficiency of intercellular adhesion molecule 1 (ICAM-1), an established ligand of Mac-1, did not impair macrophage fusion, suggesting the involvement of other counter-receptors. Here, using various approaches, including signal regulatory protein α (SIRPα) knockdown, recombinant proteins, adhesion and fusion assays, biolayer interferometry, and peptide libraries, we show that SIRPα, which, similar to ICAM-1, belongs to the Ig superfamily and has previously been implicated in cell fusion, interacts with Mac-1. The following results support the conclusion that SIRPα is a ligand of Mac-1: (a) recombinant ectodomain of SIRPα supports adhesion of Mac-1–expressing cells; (b) Mac-1–SIRPα interaction is mediated through the ligand-bind- ing α5β1-domain of Mac-1; (c) recognition of SIRPα by the α5β1-domain conforms to general principles governing binding of Mac-1 to many of its ligands; (d) SIRPα reportedly binds CD47; however, anti-CD47 function-blocking mAb produced only a limited inhibition of macrophage adhesion to SIRPα; and (e) co-culturing of SIRPα- and Mac-1–expressing HEK293 cells resulted in the formation of multinucleated cells. Taken together, these results identify SIRPα as a counter-receptor for Mac-1 and suggest that the Mac-1–SIRPα interaction may be involved in macrophage fusion.

Cell–cell fusion is a fundamental biological process that is required for development and homeostasis (1–3). Cellular fusion leading to the formation of multinucleated cells is essential for the formation of skeletal muscles (myoblast fusion), placental morphogenesis (fusion of trophoblast cells), fertilization (fusion of sperm and egg), and bone resorption (fusion of cells of the monocyte/macrophage lineage resulting in osteoclast generation). Cell fusion may also play a role in a number of pathological conditions. In particular, in contrast to most fusion cell types, which undergo fusion as a part of their normal development program, fusion of macrophages leading to the formation of multinucleated giant cells (MGCs) is a common feature of many granulomatous infections (4). It also accompanies chronic inflammatory conditions, including foreign body reactions to implanted biomaterials, rheumatoid diseases, giant cell arteritis, and others (4, 5). The functional role of macrophage fusion in these diseases remains unclear, but recent studies favor the idea that the increased membrane area arising from multinucleation facilitates phagocytosis of large particles and thus the removal of debris from tissues (6).

Although the molecular mechanisms of intracellular membrane fusion mediated by SNAREs and virus–host cell fusion have been established, the processes that mediate fusion of the plasma membranes, including those of macrophages, remain poorly understood (7). Consistent with the requirement for cell–cell contacts as an obligatory step in fusion, several receptors on the surface of macrophages, including SIRPα (MFR), CD47, CD44, E-cadherin, tetraspanins, DC-STAMP, and others, have been shown to participate in this process (2). However, the counter-receptors for these molecules, perhaps with the exception of E-cadherin, which mediates homophilic interactions, are poorly characterized. Previous studies demonstrated that integrin Mac-1 (α5β2β2, CD11b/CD18, and CR3), a multiligand receptor abundantly expressed on the surface of macrophages, is essential for MGC formation in vitro inasmuch as IL-4–induced fusion of Mac-1–deficient macrophages was reduced (8, 9). The examination of adhesive reactions known to be required for fusion showed that only macrophage spreading, but not adhesion to Permanox plastic, a surface permissive for fusion, was reduced in Mac-1–deficient cells (9). Furthermore, migration of IL-4–induced WT and Mac-1–deficient macrophages

This article contains Figs. S1–S6.

1 The abbreviations used are: MGC, multinucleated giant cell; DMEM, Dulbecco’s modified Eagle’s medium; SIRPα, signal regulatory protein α; m, mouse; h, human; MFR, macrophage fusion receptor; Ni-NTA, nickel-nitrilotriacetic acid; HBSS, Hank’s balanced salt solution; PMA, phorbol 12-myristate 13-acetate; BLI, biolayer interferometry; PVP, polyvinylpyrrolidone; DAPI, 4',6-diamidino-2-phenylindole.

© 2019 Podolnikova et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.
phages was similar (9). Although Mac-1–initiated signaling leading to cytoskeletal rearrangements and cell spreading may be critical early events during macrophage fusion, Mac-1 may fulfill other functions.

Macrophage fusion requires bringing two plasma membranes together and may involve the interaction of Mac-1 with its counter-receptor(s) on opposing cells. In addition to its role in cell adhesion to the extracellular matrix, Mac-1 interacts with several counter-receptors on other cells, including ICAM-1 (10). ICAM-1 is expressed on the surface of fusing macrophages (11, 12). However, our investigations using ICAM-1–deficient murine macrophages did not support the essential involvement of this molecule in fusion (9), suggesting that Mac-1 can interact with other counter-receptor(s).

It is widely accepted that molecules containing Ig-like domains are involved in fusion. For example, recognition and adhesion between Drosophila myoblasts are mediated by Ig-domain–containing transmembrane proteins (13, 14). We have tested the hypothesis that signal regulatory protein α (SIRPα), which, similar to ICAM-1, belongs to the Ig superfamily, interacts with Mac-1. SIRPα (also known as a macrophage fusion receptor, MFR) was one of the first discovered molecules implicated in macrophage fusion (15). The experiments in this study describe the utilization of a variety of cell biology and biochemistry techniques to show that SIRPα is a ligand for Mac-1. We also provide evidence of direct interaction between the αMI-domain, a ligand-binding region of Mac-1, and the extracellular domain of SIRPα. Furthermore, we established a cell-fusion system with HEK293 cells transfected separately with Mac-1 and SIRPα to show that co-culturing these cells in the presence of IL-4 results in cell fusion.

Results

SIRPα is critical for macrophage fusion

Previous studies using mAbs raised against SIRPα expressed in rat alveolar macrophages demonstrated that SIRPα is induced by ~1.5–2-fold at the onset of fusion (15, 16) and that the recombinant ectodomain of SIRPα inhibited fusion (15), suggesting the role for this receptor in macrophage fusion. We showed that SIRPα is expressed in mouse thioglycollate-elicited peritoneal macrophages, and its expression is increased by ~1.4-fold after 6 h in culture in the presence of fusion-promoting cytokine IL-4 and is then gradually elevated (~1.7-fold) until 48 h (Fig. S1, A and B). To substantiate the role of SIRPα in fusion, we performed knockdown (KD) of SIRPα in a RAW264.7 macrophage cell line using shRNA. Stable clones expressing shRNAs were selected, expanded, and sorted to obtain cells with no SIRPα expression (Fig. 1A). Macrophage fusion was induced by IL-4 on fusion-permissive Permanox dishes. As shown in Fig. 1, B and C, fusion of selected SIRPα-KD cells was efficiently ablated compared with wildtype (WT) RAW264.7 cells and cells transduced with control GFP-expressing viruses. Macrophage fusion is known to involve adhesion of cells to the surface (2). Consequently, we determined whether the procedure affected the adhesive properties of cells. We showed that SIRPα-KD cells adhered to Permanox to a similar extent as control cell (Fig. 1D). Because macrophages move during fusion in vitro (2, 9), we also examined whether SIRPα-KD cells have different migratory behavior during IL-4–induced fusion. Using live-cell microscopy, we found no difference in the rate of migration of control and SIRPα-KD cells (Fig. 1E). We recently showed that integrin Mac-1 is required for macrophage fusion induced by IL-4 (9). To determine whether knockdown of SIRPα affected expression of Mac-1 on the surface of RAW264.7, we performed FACS analyses using mAb 44a against the αMI integrin subunit. As shown in Fig. 1F, similar mean fluorescence intensity signals were obtained using selected SIRPα-KD and lentivirus control cells (1520 ± 40 versus 1640 ± 130, respectively). Furthermore, the activation state of Mac-1 probed with an activation-dependent mAb CB1R1/5 was similar in both cell lines (Fig. 1G). These results indicate that SIRPα is a critical mediator of macrophage fusion. Furthermore, because the absence of SIRPα on the surface of macrophages does not alter the adhesive and migratory properties of macrophages, the data suggest that during fusion SIRPα may perform other functions, potentially serving as a binding partner for cell-surface molecules.

Integrin Mac-1 mediates cell adhesion to SIRPα and its individual Ig domains

To assess the possibility that SIRPα might serve as a counter-receptor for Mac-1, we expressed recombinant fragments corresponding to the entire ectodomains of homologous mouse (m) and human (h) SIRPα. In addition, we prepared three individual mSIRPα Ig-like domains or their combinations (Fig. 2A). After isolation by affinity chromatography, the fragments were characterized by size-exclusion chromatography to confirm their monomeric state (Fig. 2B, left panel). In addition, SDS-PAGE analyses provided additional verification of the proteins’ homogeneous monomeric state (Fig. 2B, right panel). When immobilized on microtiter plates, both mouse and human SIRPα fragments (termed Ig1-2-3) supported efficient adhesion of Mac-1–expressing HEK293 cells (Mac-1–HEK293), a cell line often used for investigations of the capacity of proteins to serve as Mac-1 ligands (Fig. 3, A and C) (17, 18). By contrast, no adhesion of WT HEK293 cells (WT HEK293) was observed (Fig. 3, A and C). Adhesion of Mac-1–HEK293 cells to SIRPα was similar to that of fibrinogen, a well-characterized Mac-1 ligand (Fig. S2) (19, 20). In agreement with the involvement of Mac-1 in binding to SIRPα, function-blocking mAb 44a against the αMI integrin subunit of Mac-1, but not an isotype control antibody, inhibited adhesion of Mac-1–HEK293 cells to both mouse and human proteins (Fig. 3, B and C). The specificity of the interaction was further confirmed using HEK293 cells expressing the related monospecific β2 integrin αMI (LFA-1); no cell adhesion to SIRPα was observed (Fig. 3C). The interaction between Mac-1 and hSIRPα was mediated by the αMI-domain of Mac-1 inasmuch as cells expressing the “I-less” form of the receptor were incapable of supporting adhesion (Fig. 3C). The lack of adhesion of LFA-1- and I-less–Mac-1–HEK293-expressing cells to SIRPα was not due to the different density of receptors because, as assessed by flow cytometry, both cell lines expressed the same levels of the β2 integrin subunit as Mac-1–HEK293 cells (Fig. S3). The ability of individual Ig domains of mSIRPα (Ig1, Ig2, and Ig3) or their combinations (Ig1-2 and
Ig2-3) to support adhesion of Mac-1–HEK293 cells was also examined. As shown in Fig. 3D, all fragments were capable of supporting concentration-dependent cell adhesion, albeit to a different extent.

Because adsorption of proteins on plastic leads to their unfolding, which may result in the exposure of cryptic Mac-1–binding sites, we examined whether soluble SIRPα-derived fragments can interact with Mac-1. Mac-1–HEK293 cells were preincubated with 1.5 μM of each soluble fragment and then added to the wells coated with mSIRPα Ig1-2-3. All of the tested fragments significantly decreased cell adhesion (Fig. 3E), with Ig2-3 and Ig3 being most active (92 ± 2 and 84 ± 3% of inhibition, respectively). It was shown that SIRPα can dimerize in cis on the cell surface with all three Ig extracellular domains being implicated in the complex formation and with N-linked protein glycosylation playing some role in dimerization (21). Although our recombinant fragments were produced in *Escherichia coli* cells that lack this modification, we nevertheless examined whether the inhibitory effect of individual domains on cell adhesion might have arisen from complex formation between the immobilized mSIRPα Ig1-2-3 and soluble fragments, resulting in masking of the Mac-1–binding site(s). In these experiments, the Ig1-2-3 protein immobilized onto microtiter wells was first preincubated with 1.5 μM of each fragment, after which cells were added. In contrast to the experimental format in which cells were initially preincubated with the fragments (Fig. 3E), little inhibition was observed (Fig. S4), suggesting that the observed blocking of adhesion was mainly due to the interaction between Mac-1 and soluble SIRPα fragments.

We next assessed the ability of the mouse macrophage cell line RAW264.7 and mouse peritoneal inflammatory macrophages naturally expressing Mac-1 to interact with mSIRPα. As
**SIRPα is a counter-receptor for integrin Mac-1**

Figure 2. Characterization of SIRPα-derived recombinant fragments. A, schematic representation of the domain structure of mouse SIRPα showing the N- and C-terminal residues of the constituent Ig1, Ig2, and Ig3 domains. B, HPLC elution profiles and SDS-PAGE analyses of the purified mouse (mSIRPα) and human (hSIRPα) proteins. Proteins were purified from soluble fractions of E. coli lysates by metal-affinity chromatography using Ni-NTA–agarose column (Qiagen) followed by purification using high-performance size-exclusion chromatography on the TSKgel G3000 SW resin. The retention times of the protein peaks were compared with those of standard proteins (thyroglobulin, 670 kDa; γ-globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; and vitamin B12, 1.35 kDa). The areas shaded in gray in the chromatograms for mSIRPα Ig1-2-3, mSIRPα Ig2-3, and mSIRPα Ig1-2 denote the fractions used for experiments. Recombinant proteins were characterized by SDS-PAGE. M, markers; NR, nonreduced samples; R, reduced samples.

shown in Fig. 4A, PMA-stimulated RAW264.7 macrophages and peritoneal macrophages adhered to mSIRPα Ig1-2-3 in a concentration-dependent manner. The mAb M1/70 directed to the mouse αM integrin subunit inhibited adhesion of RAW264.7 cells by 80 ± 2%, suggesting that Mac-1 is involved in adhesion to mSIRPα (Fig. 4B). The specificity of M1/70’s effect was established using rat IgG2b, an isotype control for mAb M1/70. Preincubation of RAW264.7 cells with this IgG did not inhibit cell adhesion (Fig. 4B). Partial inhibition of adhesion by mAb M1/70 suggested that in addition to Mac-1, other structures on the surface of macrophages may be involved in binding to mSIRPα. To investigate this possibility, we examined whether heparan sulfate and chondroitin sulfate proteoglycans, which commonly cooperate with Mac-1 in adhesion to its ligands (22–25), are involved in adhesion to SIRPα. Whereas preincubation of RAW264.7 cells with 10 μg/ml of each heparin, chondroitin sulfate A, and chondroitin sulfate B partially inhibited adhesion (35 ± 1, 47 ± 8, and 28 ± 3%, respectively), their combination with mAb M1/70 did not potentiate the inhibitory effect of the mAb (Fig. 5S). Some involvement of CD47, a known ligand for SIRPα (26), was noted as mAb miap301 against mouse CD47 inhibited adhesion by 29 ± 5%, whereas an isotype control IgG2a inhibited adhesion by ~5% (Fig. 4B). However, the combination of anti-CD47 and M1/70 did not produce inhibition greater than that caused by mAb M1/70 alone. Similar to RAW264.7 cells, anti-Mac-1 and anti-CD47 mAbs partially inhibited adhesion of mouse peritoneal macrophages (by 56 ± 2 and 46 ± 4%, respectively) (Fig. 4C). However, in these cells, the effect of anti-Mac-1 was less pronounced, whereas anti-CD47 inhibited adhesion to a greater extent than in RAW264.7 cells. Furthermore, the combined effect of two antibodies was slightly, albeit significantly, greater (66 ± 2%) than that caused by anti-Mac-1 mAb M1/70 alone, suggesting that both receptors may contribute to adhesion. Together, the cell adhesion data identify the Mac-1 integrin on the surface of macrophages as a receptor for SIRPα. They also suggest that Mac-1 may cooperate with CD47.

**Mac-1 and CD47 form a complex on the surface of Mac-1-HEK and RAW264.7 cells**

Because anti-CD47 mAb partially decreased adhesion of RAW264.7 cells to mSIRPα (Fig. 4C), we examined the contribution of CD47 to adhesion of Mac-1-HEK293 cells, which also express this cell-surface protein (Fig. 5A, left panel). In these experiments, we used mAb B6H12, which reportedly inhibited adhesion of CD47 on erythrocytes to the immobilized recombinant ectodomain of hSIRPα (27). Although inhibition of cell adhesion to hSIRPα by mAb B6H12 was detected, the effect appeared to be nonspecific because an isotype IgG1 control decreased adhesion to a similar extent (46 ± 6% versus 42 ± 5%) (Fig. 5B). Furthermore, consistent with the lack of specific inhibition, B6H12 also decreased cell adhesion to mSIRPα, which
SIRPα is a counter-receptor for integrin Mac-1

**Figure 3. SIRPα supports adhesion of Mac-1–expressing HEK293 cells.** A, adhesion of Mac-1–HEK293 and WT HEK293 cells to mSIRPα. Microtiter wells were coated with different concentrations of mSIRPα Ig1-2-3 followed by post-coating with PVP. Aliquots of cells (5 × 10^4/0.1 ml) were added to microtiter wells for 30 min at 37 °C. Nonadherent cells were removed, and the number of adherent cells was determined by measuring the fluorescence. B, inhibition of adhesion of Mac-1–HEK293 cells to mSIRPα Ig1-2-3 by anti-mAb 44a. Mac-1–HEK293 cells were incubated with increasing concentrations of mAb 44a before adding to the wells coated with 5 μg/ml mSIRPα. Mouse IgG1 (G3A1) was used as an isotype control. C, adhesion of WT, Mac-1–, I-less Mac-1–, and LFA-1-expressing HEK293 cells to the wells coated with 5 μg/ml hSIRPα Ig1-2-3. The effect of anti-mAb 44a (10 μg/ml) is also shown. D, microtiter wells were coated with different concentrations of mSIRPα 1-2-3 or truncated proteins (Ig1, Ig1-2, Ig2-3, and Ig3), and adhesion of Mac-1–HEK293 cells was determined. E, inhibition of adhesion of Mac-1–HEK293 cells to immobilized mSIRPα Ig1-2-3 (5 μg/ml) by soluble mSIRPα-derived truncated proteins. Prior to adhesion, cells were incubated with 1.5 μM of each soluble protein. Data in A and D are expressed as percent of added cells. Data in B and E are expressed as percent of control adhesion without inhibitors. Data in C are expressed as percent of adhesion mediated by Mac-1–HEK293 cells. The data shown are means ± S.E. of two to five individual experiments performed with three to six determinations at each experimental data point. **, p < 0.01; ***, p < 0.001.

does not interact with human CD47 (Fig. 5B) (28). These data suggested that CD47 expressed on Mac-1–HEK293 cells was not involved in the interaction with hSIRPα during adhesion. The lack of interaction between CD47 and hSIRPα is in agreement with complete inhibition of adhesion of these cells by anti-Mac-1 mAb 44a and consistent with the inability of SIRPα to support adhesion of WT HEK293 cells (Fig. 3, A and B).

Nevertheless, the specific effect of anti-CD47 mAbs on adhesion of RAW264.7 macrophages (Fig. 4C) suggested that Mac-1 and CD47, which are present on these cells (Fig. 5A, right panel), may separately interact with different SIRPα molecules. Alternatively, Mac-1 can form a lateral complex with CD47, which may engage SIRPα. CD47 was shown to interact with β1 and β3 integrins (29) and proposed to interact with β2 integrins.
SIRPα is a counter-receptor for integrin Mac-1

Figure 4. Adhesion of RAW264.7 cells and mouse peritoneal macrophages to mSIRPα. A, adhesion of RAW264.7 and peritoneal mouse macrophages to microtiter wells coated with different concentrations of mSIRPα lg1-2-3. B, effect of anti-αM mAb M1/70, its isotype control rat IgG2b, anti-CD47 mAb miap301, and its isotype control IgG2a on adhesion of RAW264.7 cells to mSIRPα lg1-2-3 immobilized at 2.5 µg/ml. Prior to adhesion, RAW264.7 cells (5 × 10⁷/0.1 ml) were incubated with 10 µg/ml each mAb or their mixture for 15 min at 22 °C. Data are expressed as a percentage of control adhesion in the absence of added reagents and are mean ± S.E. of three to six individual experiments performed with triplicate determinations at each experimental data point. C, effect of M1/70 and anti-CD47 mAbs on adhesion of mouse peritoneal macrophages to microtiter wells coated with 2.5 µg/ml mSIRPα lg1-2-3. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, nonsignificant difference.

As shown previously, within its ligands the αM-I-domain can bind short sequences enriched in basic and hydrophobic residues (34). To examine whether the binding specificity of Mac-1 toward SIRPα conforms to this recognition principle, we analyzed the sequences of the Ig domains of hSIRPα by the computer program that determines the capacity of 9-mer peptides spanning the sequences of Mac-1 ligands to interact with the αM-I-domain (34). It assigns each peptide the energy value that serves as a measure of probability for the αM-I-domain that binds this sequence: the lower the energy, the higher the likelihood of binding. To corroborate these data, we analyzed the binding parameters of the interaction between the αM-I-domain and hSIRPα by biolayer interferometry (BLI). Because αM-I-domain can exist in two different states, active and inactive, with the length of the C-terminal α-helix regulating its activation state (31–33), we examined both conformers. Soluble lg1-2-3 ectodomain of hSIRPα was coupled to the matrix on the biosensor, and the binding of αM-I-domains was measured in buffer containing 1 mM MgCl₂. Fig. 6A shows that active αM-I-domain bound to SIRPα in a dose-dependent manner. The Kₜₐ value for the binding was found to be 1.4 µM. As a specificity control, the interaction was inhibited by mAb 44a directed to the αM-I-domain (Fig. 6B). The inactive form of the αM-I-domain minimally bound SIRPα (Fig. 6B). In contrast to the active αM-I-domain, the active form of αI-I-domain derived from the monospecific integrin αIβ₂ (LFA-1) did not interact with SIRPα. These results indicate that similar to many other ligands, the binding of Mac-1 to SIRPα is mediated by αM-I-domain, and this interaction requires the active state of αM-I-domain.

Identification of αM-I-domain–binding sequences in the extracellular Ig domains of SIRPα

As shown previously, within its ligands the αM-I-domain can bind short sequences enriched in basic and hydrophobic residues (30). However, the latter interaction was not documented. To investigate the latter possibility, we immunoprecipitated Mac-1 from RAW264.7 and Mac-1–HEK293 cells using mAbs against the corresponding αM-I integrin subunits and analyzed immune complexes for the presence of CD47. As shown in Fig. 5C, mAbs 44a and M1/70 precipitated the αM and β₂ integrin subunits from Mac-1–HEK293 and RAW264.7 cells, respectively, as well as a protein with a molecular mass of 47 kDa. Western blotting analyses confirmed the identity of this protein as CD47 in the precipitates from both types of cells (Fig. 5D). Conversely, anti-CD47 mAbs immunoprecipitated αM and β₂ integrin subunits (Fig. 5C). Control immunoprecipitations performed with isotype-specific IgGs for mAbs against human and mouse antigens apparently did not pull down proteins (Fig. 5, C and D). These experiments suggest that Mac-1 forms a lateral complex with CD47, which potentially may engage the SIRPα molecule(s) on the opposite cell.
**Figure 5.** Mac-1 associates with CD47 on the surface of RAW264.7 and Mac-1–expressing cells. A, flow cytometry analysis of surface expression of CD47 on RAW264.7 and Mac-1–HEK293 cells. The Mac-1–HEK293 cells were incubated with mouse anti-CD47 mAb B6H12.2 (gray area), mouse IgG1 isotype control G3A1 (solid line), and Alexa Fluor 488–conjugated secondary antibody (dashed line). RAW264.7 cells were incubated with rat anti-CD47 mAb miap301 (gray area), rat IgG2a isotype control antibody (solid line), and Alexa Fluor 488–conjugated secondary antibody (dashed line). B, effect of anti-CD47 mAb B6H12.2 and mouse IgG1 isotype control on adhesion of Mac-1–HEK293 cells to wells coated with 5 μg/ml mouse (right panel) or human (left panel) SIRPα Ig1-2-3. Data are expressed as a percentage of control adhesion in the absence of antibodies and are mean ± S.E. of three to four individual experiments performed with triplicate determinations in each experiment. *, p < 0.05 versus control Mac-1–HEK293 cells. C and D, RAW264.7 and Mac-1–HEK293 cells were biotinylated and immunoprecipitated (IP) with mAbs against the αM-subunit of Mac-1 or CD47. Blots were disclosed with streptavidin-alkaline phosphatase (C), or analyzed with mAb against CD47 (D). WB, Western blot.

*SIRPα is a counter-receptor for integrin Mac-1*
SIRPα is a counter-receptor for integrin Mac-1

Figure 6. Analyses of binding of the αMI-domains to SIRPα Ig1-2-3 by BLI. A, representative binding curves of titration of hSIRPα Ig1-2-3 immobilized on the FortéBio sensor with active αMI-domain (0.1–3.5 μM). RU, response units. Inset, saturable binding curve of αMI-domain binding to SIRPα Ig1-2-3. Req, response at equilibrium. B, comparison of different I-domain fragments for their ability to bind SIRPα. Data are expressed as a percentage of control binding of active αMI-domain and are mean ± S.E. of three individual experiments. ***, p < 0.001.

To demonstrate the importance of the identified SIRPα sequences for interaction with Mac-1, we examined the ability of hSIRPα-derived peptides to support Mac-1–mediated cell adhesion. In these experiments, we synthesized selected peptides that correspond to the αMI-domain–binding sequences in each Ig domain and that are fully exposed on the surface of the protein (Fig. 7C): 36IQWFRGAGP44 (termed PD1), 192ITLKWFKNG158 (termed PD2), and 245KVFYPCRQL253 (termed PD3). As shown in Fig. 7D, all three peptides supported efficient adhesion of Mac-1–HEK293 cells, whereas the control peptide 161SDFQTNVD169 (13.2 kJ/mol) was negative. We further tested the ability of peptides to block cell adhesion to hSIRPα. Preincubation of cells with soluble peptides resulted in a dose-dependent inhibition of adhesion (data not shown). At 100 μg/ml (maximal testable concentration), all peptides decreased adhesion by ~50% (Fig. 7E), whereas the control peptide had no effect. These findings indicate that similar to other Mac-1 ligands, the αMI-domain recognizes the SIRPα peptides enriched in basic and hydrophobic residues and suggests that all three Ig domains contain putative αMI-domain–binding sites.

Reconstitution of cell–cell fusion in the mixture of Mac-1- and SIRPα-expressing HEK293 cells

Previous studies demonstrated that HEK293 cells stably transfected with the P2X7 receptor cDNA can fuse forming multinucleated cells (35). We used HEK293 cells to examine the hypothesis that the interaction between Mac-1 and SIRPα can promote fusion. The hSIRPα was stably expressed in HEK293 cells (Fig. 8A), and these cells were mixed with Mac-1–HEK293 cells at 1:1, 1:2, and 1:4 ratios. Cells were plated on Permanox dishes, and IL-4 was added to the mixture for 72 h. As shown in Fig. 8B, cell fusion was detected (three representative images are shown). Although these events were rare (~3–4%, n = 4) at a 1:1 SIRPα–HEK293/Mac-1–HEK293 cell ratio, the fraction of fused multinucleated cells increased to 15 ± 3% (n = 3) at a 1:4 ratio. In contrast, fused cells were never observed in the populations of IL-4–treated WT HEK293 cells or among cells expressing either Mac-1 or SIRPα alone (Fig. 8C). It is unlikely that multinucleated cells in the mixture of Mac-1- and SIRPα-expressing cells originated from karyokinesis in the absence of cytokinesis, as some cells contained 6–20 nuclei. Furthermore, confocal images of multinucleated cells stained with anti-αMI mAb 44a showed the presence of two labels, Mac-1 (red) and endogenous GFP (co-expressed with SIRPα), which is only possible if SIRPα-expressing cells fused with Mac-1–expressing cells (Fig. 8D). In contrast, individual Mac-1–HEK293 and SIRPα–HEK293 cells (not treated with IL-4) were seen as small mononucleated cells stained with either red or green labels (Fig. 8D, bottom panel). In addition, the presence of two labels was...
Figure 7. Identification of putative α4β1-domain–binding sites in hSIRPα. A, peptide library derived from the sequence of human SIRPα consisting of 9-mer peptides with a three-residue offset. The peptide energies that serve as a measure of probability each peptide can interact with the α4β1-domain were calculated as described (34) and are shown on the right of each column. The predicted α4β1-domain–binding sequences with the lowest energies are highlighted. B, library assembled on the cellulose membrane was incubated with 125I-labeled peptides with a three-residue offset. The peptide energies that serve as a measure of probability each peptide can interact with the MI-domain of hSIRPα based on Protein Data Bank code 2WNG (37). The α4β1-domain–binding sequences exposed on the surface of each Ig domain of SIRPα are colored in red, green, and blue. C, 3D structure of the extracellular domain of hSIRPα consisting of 9-mer peptides were coated on microtiter wells followed by post-coating with 1% PVP. Adhesion of Mac–1–HEK293 cells was determined as described under "Experimental procedures." ***, p < 0.001; ns, nonsignificant difference.
detected in multinucleated cells that formed in the mixture of SIRPα–HEK293 cells (green) and Mac-1–HEK293 cells transfected with mCherry (red) (Fig. 8E).

Having established conditions for the induction of HEK293 cell fusions, we examined the fusion-promoting capacity of individual SIRPα Ig domains. The SIRPα proteins lacking the Ig1 (termed hSIRPα 2–3) and Ig1-2 (termed hSIRPα 3) extracellular domains were expressed on the surface of HEK293 cells (Fig. 8A), and their fusion was compared with that of cells expressing the entire SIRPα ectodomain. The HEK293 cells expressing WT and SIRPα mutants were mixed with Mac-1–HEK293 cells at a 1:4 ratio and allowed to fuse for 72 h. Fig. 8F shows that the fusion index of cells expressing hSIRPα-Ig2-3 was lower than that of WT protein. In contrast, cells expressing hSIRPα-Ig3 fused somewhat better than WT cells. Although the trend in both cases was clearly seen, the difference between WT and mutant cells did not reach a statistical significance. Nevertheless, the fusion rate of cells expressing only Ig3 was significantly higher than that of cells expressing Ig2-3. Although the interpretation of these results is difficult, the higher fusion-promoting capacity of Ig3 is in agreement with its higher activity in blocking the interaction of Mac-1–HEK293 cells with SIRPα in adhesion assays (Fig. 3E). Furthermore, the ability of Ig3 alone to support fusion further suggests that on the surface of Mac-1–HEK293 cells, CD47 is not involved in the interaction with SIRPα because it reportedly interacts with Ig1 of SIRPα (16, 36, 37).

Discussion

In a previous in vitro study, we established that integrin Mac-1 plays an essential role in macrophase fusion (9). We also demonstrated that macrophages lacking ICAM-1, a counter-receptor for Mac-1, undergo fusion to a similar extent as WT cells implicating an unknown molecule(s) on the surface of fusing macrophages as a counter-receptor for this integrin. The major finding of this study is that SIRPα serves as a heretofore unrecognized ligand for Mac-1. The following data support this conclusion. First, the recombinant ectodomain of SIRPα encompassing three Ig-like domains (Ig1-2-3) supports adhesion of Mac-1–expressing HEK293 cells as well as natural macrophages. Second, the functional region of Mac-1, which mediates the interaction with SIRPα, is the αM domain. Third, recognition specificity of the αM domain toward SIRPα conforms to the expected pattern exhibited by many Mac-1 ligands. Fourth, co-culturing of HEK293 cells separately expressing Mac-1 and SIRPα results in cell fusion and formation of multinucleated cells, whereas no fusion is observed in cell populations expressing only one of these receptors. Fifth, although CD47 was previously reported to bind SIRPα, no significant involvement of CD47 in the binding of Mac-1–expressing cells to SIRPα was detected: instead, CD47 was found to form a lateral complex with Mac-1.

Integrin Mac-1 is a major adhesion receptor on the surface of myeloid cells. This receptor exhibits multitiligand binding properties enabling it to bind a variety of proteins in the extracellular matrix as well as numerous cationic proteins released from stimulated neutrophils and damaged cells during the inflammatory response (18, 22, 34). In addition, Mac-1 can bind counter-receptors expressed on a number of cells, including ICAM-1, GPRh-IX, and JAM-3 (10, 38, 39). Two of these molecules, ICAM-1 and JAM-3, contain multiple extracellular Ig-like domains. It has been noted that the molecules containing Ig-like domains widely participate in fusion events mediating cell–cell tethering (14). For example, recognition and adhesion between Drosophila melanogaster myoblasts are mediated by Ig-like domain-containing transmembrane proteins DUF, RST, and SNS (1, 14). Similar to ICAM-1, SIRPα is a transmembrane protein that contains three extracellular Ig domains (the N-terminal IgV domain followed by two IgC domains). A shorter variant of SIRPα lacks the C1 domains and contains only the IgV domain. SIRPα, also known as MFR, was identified initially by antibodies that blocked fusion of rat alveolar macrophages and was shown to be up-regulated after 24 h in culture under fusogenic conditions (40). We found that SIRPα is also up-regulated on the surface of IL-4–treated mouse peritoneal macrophages (Fig. S1). To test Mac-1–SIRPα binding, we initially characterized the interaction between the Mac-1–expressing cells and the ectodomain of SIRPα (Ig1-2-3) using adhesion assays. Both human and mouse Mac-1–expressing cells supported efficient Mac-1–dependent adhesion to immobilized SIRPα. We found that all three Ig-like domains of SIRPα have the capacity to interact with Mac-1, with Ig3 being the most active. However, although Mac-1 appears to be the main receptor for SIRPα on the surface of Mac-1–expressing HEK293 cells (Fig. 3), CD47 is not involved in the surface of RAW264.7 cells and mouse peritoneal macrophages may contribute to adhesion (Fig. 4, B and C).

Because immobilization of proteins on plastic may result in their partial denaturation resulting in exposure of hidden interior sequences that are favored by Mac-1 (41), we have also tested the interaction of Mac-1–expressing cells with soluble SIRPα fragments. The SIRPα ectodomain, as well as recombinant fragments duplicating its constituent Ig-like domains, blocked cell adhesion (Fig. 3E), suggesting that Mac-1 may bind intact SIRPα expressed on the cell surface. The activity of soluble fragments in inhibition adhesion assay appears to closely correspond to their capacity to support Mac-1–mediated cell adhesion, with Ig3 again being most potent. Furthermore, the interaction between SIRPα and the recombinant ligand-binding αM domain of Mac-1 has been detected using BLI. In this system, SIRPα Ig1-2-3 was immobilized to a dextran-coated surface by chemical cross-linking, a procedure that largely preserves the native protein conformation. Based on these results, we propose that SIRPα is a novel counter-receptor of Mac-1 on the surface of macrophages involved in cell–cell interactions.

The characteristic feature of some Mac-1 ligands is the presence of short sequences containing basic residues surrounded by hydrophobic residues, which form αM domain recognition motifs (34). Screening of the peptide library spanning the sequence of hSIRPα Ig1-2-3 revealed that it contains several typical αM domain recognition motifs in all three Ig domains. The ability of selected SIRPα-derived peptides to interact with Mac-1 in adhesion and inhibition adhesion assays (Fig. 7) recapitulates the behavior of other well-characterized Mac-1 peptide ligands (23, 24, 42, 43). Consistent with the presence of multiple recognition sites in Mac-1 ligands (34, 44), the αM domain–binding sequences have been found in all three Ig
**Figure 8.** Formation of multinucleated cells in the mixture of Mac-1- and SIRPα-expressing HEK293 cells.  

A, flow cytometry analyses of surface expression of Mac-1, full-length hSIRPα, and truncated SIRPα proteins on the surface of HEK293 cells. The cells were incubated with mAb 44a (anti-αM) followed by Alexa Fluor 488–conjugated secondary antibody or SE7C2 (anti-hSIRPα) followed by Alexa Fluor 633–conjugated secondary antibody. B, Mac-1- and SIRPα–HEK293 cells were suspended at 1 × 10^6/ml and mixed at a 4:1 ratio, and 0.5 ml of the mixture was plated on Permanox dishes in the presence of IL-4. After 72 h, cell cultures were fixed and stained with Wright stain. Three representative images from seven separate experiments are shown. Scale bar, 50 μm. C, control WT HEK293, Mac-1–HEK293, and SIRPα–HEK293 cells were plated at 5 × 10^4/0.5 ml on Permanox dishes and cultured in the presence of IL-4 for 72 h. Representative images of cell cultures are shown. Scale bar, 10 μm. D, upper panel: Mac-1–HEK293 and SIRPα–HEK293 (expressing endogenous GFP) cells were mixed at 4:1 and plated on glass slides in the presence of IL-4. After 72 h, cells were fixed and incubated with mAb 44a (anti-αM) followed by secondary Alexa Fluor 633–conjugated antibody. Arrowheads indicate the location of green puncta in the red Mac-1–expressing cell. Bottom panel: two representative mononuclear cells expressing SIRPα with endogenous GFP (green) or Mac-1 stained with mAb 44a (red). These cells were not treated with IL-4. Scale bar, 10 μm. E, Mac-1–HEK293 cells were transiently transfected with mCherry and mixed with SIRPα–HEK293 cells (4:1). Cells were plated on glass, and fusion was induced with IL-4 for 72 h. Upper panel, bright field image of the multinucleated cell (left), staining with DAPI (middle), and a composite image of the same field (right). Scale bar, 10 μm. Bottom panel, multinucleated cell showing the distribution of SIRPα, Mac-1, and both green and red labels. F, Mac-1–HEK293 cells were mixed with HEK293 cells expressing the Ig2-3 and Ig3 of SIRPα at a 4:1 ratio, plated on Permanox dishes, and fusion-induced with IL-4 for 72 h. The fusion index was determined after staining of cultures with Wright stain. *, p < 0.05; ns, nonsignificant difference. MFI, mean fluorescence intensity.
domains of SIRPα (Fig. 7C). The greater activity of SIRPα Ig3 in adhesion (Fig. 3, D and E) and fusion assays (Fig. 8F) seems to implicate this domain as a major site in Mac-1 binding, which may be attributable to the presence of an extended cluster of the αMβ2-domain–binding sequences containing RKFYQRLQ, the most active peptide in the SIRPα-derived peptide library (Fig. 7A).

Previous studies showed that CD47, a widely distributed plasma membrane protein, is a ligand for SIRPα (26, 29). Furthermore, the recombinant soluble ectodomain of CD47 blocked fusion of rat alveolar macrophages in culture, suggesting the involvement of CD47 in macrophage fusion (16). CD47, which also belongs to the Ig superfamily, contains one N-terminal extracellular variable Ig domain (IgV) followed by five transmembrane segments. Because the recombinant IgV domain of CD47 binds both forms of SIRPα, long and short, it has been proposed that the IgV domain of CD47 interacts with the IgV domain of SIRPα (16). The interaction between the IgV domains of these two molecules has been confirmed by solving the three-dimensional structure of their complex (37). The lack of adhesion of HEK293 cells, which express CD47, to the ectodomain of SIRPα in our experiments and the finding that adhesion of Mac-1–expressing HEK293 cells was completely inhibited by anti-Mac-1 mAb, but not by anti-CD47 blocking antibody, suggest that in these cells CD47 is not involved in SIRPα binding. The SIRPα–CD47 interactions exhibit little cross-reactivity across species (27). In this regard, human SIRPα does not bind mouse CD47 (27), and mouse SIRPα does not significantly interact with human CD47 (28). Therefore, the finding that both mouse and human SIRPα supported adhesion of Mac-1–HEK293 cells (Fig. 3) seems to further support the CD47-independent binding of these cells to SIRPα. Nevertheless, on the surface of RAW264.7 and mouse peritoneal macrophages, CD47 may potentially contribute to binding to SIRPα, because the antibody specific for mouse CD47 (miap301) partially blocked cell adhesion to mouse SIRPα (Fig. 4, B and C). The inhibitory effect of both anti-Mac-1 and anti-CD47 mAbs suggests a functional relationship between Mac-1 and CD47.

CD47 has been shown to interact with β1 and β3 integrins, including αβ2, αMβ2, and αβ1, and to propose to interact with β2 integrins (30). However, the latter interaction has not been documented. We show for the first time that anti-Mac-1 mAbs immunoprecipitated CD47 from Mac-1–expressing HEK293 cells and RAW264.7 macrophages and, conversely, the mAbs directed to CD47-immunoprecipitated Mac-1 (Fig. 5). This indicates that similar to other integrins, Mac-1 interacts with CD47 in cis (schematically shown in Fig. 9). This also suggests that this association may influence the function of integrin. Our recent studies showing that IL-4–induced macrophage fusion is most efficient when Mac-1 is present on each fusion partner may provide indirect support for this idea (9). In particular, we showed that the ability of WT macrophages to fuse with Mac-1–deficient counterparts was strongly impaired, and fusion in the mixture containing WT and Mac-1–deficient macrophages occurred mainly between WT cells. Because CD47 and SIRPα were both present on WT and Mac-1–deficient cells, where they could have mediated cell–cell interaction, these data suggest that the CD47–SIRPα interaction alone on Mac-1–deficient macrophages does not support fusion. At present, the ways by which CD47 can modify Mac-1 functions remain to be defined.

It is unclear why the interaction between Mac-1 and its counter-receptor ICAM-1 is not involved in macrophage fusion (9). ICAM-1 is expressed on the surface of mouse monocytes and macrophages, although its expression does not correlate with multinucleation (45). One possibility for the lack of involvement of ICAM-1 may be differential localization of Mac-1 and ICAM-1 on fusing macrophages. Indeed, we noted that Mac-1 and SIRPα were present at the sites of cell–cell contact between macrophages (Fig. S6A), whereas ICAM-1 was rarely observed (Fig. S6B). Furthermore, both Mac-1 and SIRPα were detected on filopodia, which may facilitate cell–cell recognition and adhesion. Macrophages express several other members of the Ig superfamily, including CD4 and CD200 and the role of CD200 in the fusion of osteoclasts, which originate from fusion of receptor activator of NF-κB ligand (RANKL)-treated macrophages as reported previously (46). Based on the resemblance of SIRPα and CD200 structures, it will be interesting to examine whether CD200 is induced in macrophages exposed to IL-4 and whether Mac-1 is capable of engaging this molecule during fusion.

Although fusion of normally nonfusing HEK293 cells after transfection with P2X7 has been shown (35), the finding of fusion of HEK293 cells after transfection with Mac-1 and SIRPα was still surprising. It has been proposed that proteins that fuse cells should fulfill several “gold standards,” including the ability to fuse heterologous cells in culture and be expressed at the time and place of fusion (47). Indeed, transfection of heterologous nonfusing insect cells with Caenorhabditis elegans fusogenic protein EFF-1 reconstituted cell fusion (7, 48). Although SIRPα is expressed at the time and place of fusion, thus obeying one of the rules, transfection of HEK293 cells with SIRPα alone was not sufficient to induce fusion (Fig. 8). It was only after
mixing SIRPα- with Mac-1–expressing HEK293 cells that fusion was detected. Yet, it is unlikely that SIRPα and Mac-1 are authentic fusogenic proteins, i.e. the proteins that mediate fusion of plasma membranes. Rather, these molecules are more likely required to bring membranes into close proximity before fusion and thus participate in the cell–cell adhesion step. If this is the case, then other undefined molecules on the surface of HEK293 cells may serve as fusogens.

In summary, we have identified SIRPα as a novel counter-receptor for Mac-1 and showed that the interaction of these molecules is mediated by the αMI-domain of Mac-1. It is interesting to discover that two molecules, Mac-1, and SIRPα that have been independently implicated in fusion are, in fact, binding partners. Based on the ability of Mac-1 and SIRPα to mediate fusion in heterologous cells, this study also proposes that the interaction between Mac-1 and SIRPα may facilitate cell–cell recognition and adhesion during macrophage fusion. Because Mac-1 forms lateral complexes with CD47, it appears that these complexes rather than individual Mac-1 molecules bind SIRPα expressed on neighboring macrophages. Furthermore, discerning the role of the Mac-1–SIRPα–CD47 complex may provide new insights into the mechanisms of macrophage fusion.

**Experimental procedures**

**Peptides, proteins, and monoclonal antibodies**

The active αMI-domain conformer (residues αMI-Glu123–Lys315) and inactive conformer with the extended C-terminal end (residues αMI-Gln119–Glu133) (33) as well as the active αI-domain (residues αI-Gly127–Tyr307, K287C, and K294C) were isolated as described previously (49). The αMI-domains were labeled with 125I using IODO-GEN (Pierce). The mouse mAb 44a directed against the human αMI integrin subunit, rat mAb M1/70 against the mouse αMI integrin subunit, and the mouse mAb B6H12 against human CD47 were purified from conditioned media of hybridoma cells obtained from the American Type Culture Collection (Manassas, VA) using protein A-agarose. The rat mAbs P84 (anti-mouse SIRPα) and M1/70 conjugated to FITC were obtained from BD Biosciences. The mouse anti-human SIRPα1 mAb (clone SE7C2) and anti-SIRP-α/β1 rabbit polyclonal antibody (H-300) were obtained from Santa Cruz Biotechnology (Dallas, TX). The rat IgG2b, an isotype control for mAbs, was obtained from eBioscience (San Diego, CA). The mouse mAb G3A1, an IgG1 isotype control for mAbs 44a and B6H12, was obtained from Cell Signaling (Beverly, MA). The anti-rat mouse CD47 mAb (clone miap301) was obtained from BioLegend (San Diego, CA). The rabbit polyclonal anti-CD18 antibody was from Proteintech (Rosemont, IL). The mAb CBRM1/5 conjugated to Alexa Fluor 488 was from Santa Cruz Biotechnology. Alexa Fluor 546–conjugated phalloidin, Alexa Fluor 488–conjugated goat anti-rat IgG, Alexa Fluor 633–conjugated goat anti-mouse IgG, and Alexa Fluor 633–conjugated goat anti-rat IgG were purchased from Life Technologies, Inc. Synthetic peptides corresponding to the 36IQWFRGAGP44 (PDI), 149ITLKWFKNL457 (PD2), 245RKFYPQRLQ253 (PD3), and 161SDFQTVNPQ169 (negative control) sequences of human SIRPα were obtained from Pep-}

宋

**Expression of recombinant mouse and human SIRPα–truncated proteins**

Constructs encoding the whole mouse or human extracellular domains of SIRPα as well as the mouse extracellular SIRPα constructs containing the individual Ig domains of SIRPα were expressed as fusion proteins with a polyhistidine tag. The coding regions for the mouse Ig-like V-type (mSIRPα Ig1, residues 32–137), mouse Ig-like V-type and C1-type 1 (mSIRPα Ig1–2, residues 32–248), mouse Ig-like C1-type 2 (mSIRPα Ig3, residues 255–343), mouse Ig-like C1-type 1 and C1-type 2 (mSIRPα Ig2-3, residues 151–356), mouse Ig-like V-type and C1-type 1 and C1-type 2 (mSIRPα Ig2-3, residues 32–248), and human Ig-like V-type and C1-type 1 and C1-type 2 (hSIRPα Ig1-2-3, residues 31–364) were amplified by PCR using as a template the full-length cDNA of mSIRPα (GenBankTM entry BC062197) and hSIRPα (GenBankTM entry BC026692), respectively. The fragments were digested with appropriate restriction enzymes and cloned in the expression vector pET28b (EMD Millipore, Billerica, MA). The accuracy of the DNA sequence was verified by sequencing. The plasmids were transformed in E. coli strain BL-21(DE3) (LysS–competent cells, and expression was induced by adding 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 37 °C. Proteins were purified from soluble fractions of E. coli lysates by metal-affinity chromatography using Ni-NTA–agarose column (Qiagen). The aggregation state of proteins was analyzed by high-performance size-exclusion chromatography using a TSKgel G3000 SW (7.5 × 30 cm) column (TOSOH Corp.). Recombinant proteins were characterized by SDS-PAGE and Western blot analysis using anti-SIRPα1 mAb SE7C2.

**Cells**

The HEK293 cells stably expressing human integrins Mac-1, αIβ2, and the “I-less form” of Mac-1 were previously described (18, 50, 51). To prepare HEK293 cells expressing human SIRPα, the full-length cDNA of human SIRPα was cloned from the Mammalian Gene Collection cDNA library (Thermo Fisher Scientific) into pAcGFP1-N1-vector (Clontech). HEK293 cells were stably transfected with the pAcGFP1-N1-SIRPα plasmid using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After 48 h at 37 °C in 5% CO2, cells were harvested and cultured in medium with 500 µg/ml G418 (Invitrogen). After 14 days, the surviving cells were collected, sorted, and analyzed using flow cytometry. The truncated SIRPα mutants with the first (SIRPα2-3) and both first and second (SIRPα3) domains deleted were produced by introducing restriction sites within the SIRPα sequence cloned into pAcGFP1-N1. The accuracy of the obtained constructs was verified by sequencing. The HEK293 cells expressing truncated SIRPα receptors were prepared as described above for the full-length SIRPα.

The mouse macrophage RAW264.7 cell line was obtained from the ATCC, and the cells were cultured in DMEM/F-12...
medium supplemented with 10% fetal bovine serum and antibiotics (0.1 mg/ml streptomycin and 0.1 unit/ml penicillin). Lentiviral particles SIRPα shRNA ((m) sc-36493) to knock down SIRPα expression in RAW264.7 cells were obtained from Santa Cruz Biotechnology. Viral particles contained three target-specific constructs that encode 19–25 nucleotides (plus hairpin) of shRNA. Stable clones expressing shRNAs were selected using puromycin dihydrochloride (2.5 μg/ml) for 4 days. Four clones were expanded and subsequently tested for SIRPα expression by FACS analysis. Thirty to 40% of the cells in each clone had SIRPα knocked down. Cells in one selected clone were sorted to obtain cells showing no SIRPα expression. As a control, shRNA lentiviral particles (sc-108080; Santa Cruz Biotechnology) containing an shRNA construct encoding a scrambled sequence were used.

Inflammatory macrophages were isolated 3 days after thioglycollate injection into the peritoneum of 8–16-week-old WT C57BL/6 and Mac-1−/− deficient mice (The Jackson Laboratory, Bar Harbor, ME) by lavage using cold PBS containing 5 mM EDTA (9). All procedures were performed in accordance with the animal protocols approved by the Institutional Animal Care and Use Committee at the Arizona State University.

Synthesis of cellulose-bound peptide libraries

The SIRPα-derived peptide library assembled on a cellulose membrane support was prepared by parallel spot synthesis (52, 53). The membrane-bound peptides were tested for their ability to bind the αMβ2-domain according to a previously described procedure (44). In brief, the membrane was blocked with 1% BSA and then incubated with 10 μg/ml of 125I-labeled αMβ2-domain in TBS containing 1 mM MgCl₂. After washing, the membrane was dried, and the αMβ2-domain binding was visualized by autoradiography.

Biolayer interferometry

The binding parameters of the interaction between the αMβ2-domain and SIRPα were determined using an Octet K2 instrument (FortéBio, Pall Corp.). The purified extracellular domain of human SIRPα was immobilized on the Amine Reactive Second-generation (AR2G) biosensor using the amine coupling kit according to the manufacturer’s protocol. Different concentrations of the active and inactive forms of αMβ2-domain and active αM1-domain were applied in the mobile phase, and the association between the immobilized and flowing proteins was detected. Experiments were performed in 20 mM HEPES, 150 mM NaCl, 1 mM MgCl₂, 0.05% (v/v) Tween 20, pH 7.5. The SIRPα-coated surface was regenerated with 25 mM NaOH. Analyses of the binding kinetics were performed using FortéBio Data Analysis 9.0 software. The value of the equilibrium dissociation constant (K_D) was obtained by fitting a plot of response at equilibrium (R_equilibrium) against the concentration.

Adhesion assays

Adhesion assays were performed as described previously (18, 50). Briefly, the wells of 96-well Immulon 4HBX polystyrene microtiter plates (Dynex Technologies, Chantilly, VA) were coated with mSIRPα or hSIRPα overnight at 4 °C. The coated wells were post-coated with 1.0% PVP for 1 h at 22 °C. Mac-1−/− expressing HEK293 cells and mouse macrophages were labeled with 10 μM calcein (Molecular Probes, Eugene, OR) for 30 min and then washed twice. RAW264.7 cells were activated with 100 nM PMA for 30 min at 37 °C at the time of labeling with calcein. Aliquots (0.1 ml) of labeled cells (5 × 10⁵/ml of HEK293 cells, 10⁵/ml of RAW 264.7 cells, and 7.5 × 10⁵/ml of mouse macrophages) in HBSS supplemented with 1 mM Ca²⁺, 1 mM Mg²⁺, and 0.1% BSA were added to each well. For inhibition experiments, cells were mixed with either antibodies or the mSIRPα recombinant proteins or peptides and incubated for 15 min at 22 °C before they were added to the coated wells. After 30 min of incubation at 37 °C, nonadherent cells were removed by two washes with PBS. Fluorescence was measured in a CytoFluorII fluorescence plate reader (PerSeptive Biosystems, Framingham, MA), and the number of adherent cells was determined by using the fluorescence of 100-μl aliquots with a known number of labeled cells.

Cell fusion

RAW264.7−, Mac-1−, and SIRPα-expressing HEK293 cells were washed and diluted at 10⁵−2.5 × 10⁵/ml in DMEM/F-12 medium, and 0.5 ml of cell suspensions were loaded into the center of 6-cm Permanox dishes (Nalge Nunc International, Rochester, NY). After 30 min of incubation, 5 ml of OptiMEM medium (Invitrogen) containing antibiotics (0.1 mg/ml streptomycin and 0.1 unit/ml penicillin) was added, and the cells were incubated at 37 °C in 5% CO₂ for 2 h. Following this, fusion was induced by adding 10 ng/ml IL-4. After incubation for 24–72 h, the dishes were washed with PBS, and cells were fixed with 3.7% paraformaldehyde followed by staining with Wright stain or incubation with Alexa Fluor 546− conjugated phalloidin. Images of representative fields were obtained using a Leica DM4000B (Leica Microsystems, Buffalo Grove, IL) microscope, and the numbers of nuclei in MGCs (=3) and mononuclear cells were counted. The extent of MGC formation was evaluated by determining the fusion index, which is defined as a fraction of nuclei within the MGCs expressed as a percentage of total nuclei counted (9). The number of visible nuclei per MGC was also counted. A total of 3−5 low-power fields (×20) containing ~100 cells was analyzed for each experimental condition.

Immunoprecipitation

Cells (5 × 10⁶) were labeled with 100 μg of Immunopure Sulfo-NHS-LC-Biotin (Pierce) in 200 μl of PBS for 30 min at 22 °C. Cells were solubilized with a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 10 μM benzamidine) for 30 min at 22 °C. The lysates were incubated with 10 μg of normal mouse IgG (Sigma) and 50 μl of Zysorbin-G (Zymed Laboratories Inc.) for 2 h at 4 °C. After centrifugation, the supernatant was incubated with 10 μg of mAb M1/70, 44a, or CD47 for 2 h at 4 °C. The immune complexes were captured by incubating with 50 μl of protein A-Sepharose (GenScript, Piscataway, NJ) for 16 h at 4 °C. The immunoprecipitated proteins were eluted with SDS-PAGE loading buffer and analyzed by Western blotting. The Immobilon-P membranes (Millipore, New Bedford, MA) were incu-
bated with streptavidin conjugated to horseradish peroxidase and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

Flow cytometry

FACS analyses were performed to assess the expression of Mac-1 and SIRPα on the surface of transfected HEK293 cells. Cells were incubated with mAb 44a (anti-α<sub>4</sub>) followed by Alexa Fluor 488–conjugated secondary antibody or SE7C2 (anti-hSIRPα) followed by Alexa Fluor 633–conjugated secondary antibody and analyzed using a FACSscan (BD Biosciences). Populations of cells expressing similar amounts of Mac-1 and SIRPα were selected by FACS using a FACS Vantage instrument (BD Biosciences). To assess expression of Mac-1, CD47, and SIRPα on the surface of RAW264.7 cells, cells were harvested, and 10<sup>5</sup> cells were incubated in 100 μl of 3% normal goat serum in 1% BSA/HBSS solution for 20 min at 4 °C. After blocking, cells were incubated in 100 μl of HBSS solution containing 10 μg/ml of respective primary antibody (anti-α<sub>4</sub>, mAb M1/70, anti-CD47 mAb miap301, and anti-SIRPα polyclonal H-300 antibody) for 30 min at 4 °C. Cells were then washed and incubated with 5 μg/ml Alexa Fluor 488–conjugated secondary antibody for an additional 30 min at 4 °C. Finally, cells were washed and analyzed as described above.

Immunofluorescence

Mouse macrophages and HEK293 cells expressing Mac-1 or SIRPα were incubated on glass slides or in Permanox chambers with 10 ng/ml IL-4 for 2–48 h and fixed in 3.7% paraformaldehyde for 20 min. Cells were incubated in a blocking buffer containing 3% normal goat serum and 1% BSA for 1 h at 22 °C. Cells were then incubated with the primary anti-mouse SIRPα mAb P84 (10 μg/ml) for 2 h at 22 °C prior to exposure to secondary anti-rat antibodies conjugated to Alexa Fluor 488 (5 μg/ml) or Alexa Fluor 633 (5 μg/ml). The second primary antibody M1/70 conjugated with FITC (10 μg/ml) was then applied in the same buffer. The rat IgG2b isotype control antibody conjugated with FITC was used as an isotype control for M1/70. Cells were mounted using Vectashield with DAPI. Confocal images were obtained using a Leica TCS SP5 AOBS Spectral Confocal microscope (Exton, PA) housed in the WM Keck Bioimaging Facility at Arizona State University.

Time-lapse microscopy and image processing

Migration of SIRPα-KD macrophages and RAW264.7 cells transduced with control lentivirus was visualized with the EVOS Live Cell imaging system (Thermo Fisher Scientific) equipped with the onset incubator that enabled control of temperature, humidity, and carbon dioxide. Macrophages were applied on Permanox slides assembled in the 4-well chamber (Nunc, Rochester, NY), and IL-4 was added after 2 h of incubation. The chambers were transferred from the CO<sub>2</sub> incubator to an onset incubator 2 h after addition of IL-4, and the recording continued for 24 h. Phase-contrast images were acquired every 30 s with the use of a ×10 objective (NA 0.25). To measure the speed of migration, 10 randomly selected cells from each cell line were tracked using ImageJ TrackMate software (National Institutes of Health, Bethesda, MD).

**Statistical analyses**

Data are presented as a mean ± S.E. The statistical comparisons between treatment groups were made using one-way analysis of variance, followed by a post hoc test (Bonferroni’s or Dunnett’s) appropriate to the analyzed data set. Statistical analyses were performed using GraphPad Prism 5 software (La Jolla, CA). A difference of p < 0.05 was considered statistically significant.

**Acknowledgments**—We thank Dr. Valeriy Lishko and Aibek Mursali-mov for technical assistance with the isolation of recombinant SIRPα fragments.

**References**

1. Chen, E. H., Grote, E., Mohler, W., and Vignery, A. (2007) Cell–cell fusion. *FEBS Lett.* 581, 2181–2193 CrossRef Medline

2. Helming, L., and Gordon, S. (2009) Molecular mediators of macrophage fusion. *Trends Cell Biol.* 19, 514–522 CrossRef Medline

3. Aguilar, P. S., Baylies, M. K., Fleissner, A., Helming, L., Inoue, N., Podbilewicz, B., Wang, H., and Wong, M. (2013) Genetic basis of cell–cell fusion mechanisms. *Trends Genet.* 29, 427–437 CrossRef Medline

4. Quinn, M. T., and Schepetkin, I. A. (2009) Role of NADPH oxidase in formation and function of multinucleated giant cells. *J. Innate Immun.* 1, 509–526 CrossRef Medline

5. Anderson, J. M., Rodriguez, A., and Chang, D. T. (2008) Foreign body reaction to biomaterials. *Semin. Immunol.* 20, 86–100 CrossRef Medline

6. Milde, R., Ritter, J., Tennent, G. A., Loesch, A., Martinez, F. O., Gordon, S., Pepys, M. B., Verschoor, A., and Helming, L. (2015) Multinucleated giant cells are specialized for complement-mediated phagocytosis and large target destruction. *Cell Rep.* 13, 1937–1948 CrossRef Medline

7. Podbilewicz, B., Leikina, E., Sapir, A., Valans, C., Suisa, M., Shenmer, G., and Chernomordik, L. V. (2006) The *C. elegans* developmental fusogen EFF-1 mediates homotypic fusion in heterologous cells and *in vivo*. *Dev. Cell* 11, 471–481 CrossRef Medline

8. Helming, L., and Gordon, S. (2007) Macrophage fusion induced by IL-4 alternative activation is a multistage process involving multiple target molecules. *Eur. J. Immunol.* 37, 33–42 CrossRef Medline

9. Podolnikova, N. P., Kushchayeva, Y. S., Wu, Y., Faust, J., and Ugarova, T. P. (2016) The role of integrins αMβ2 (Mac-1, CD11b/CD18) and αDβ2 (CD11d/CD18) in macrophage fusion. *Am. J. Pathol.* 186, 2105–2116 CrossRef Medline

10. Diamond, M. S., Staunton, D. E., de Fougerolles, A. R., Stacker, S. A., Garcia-Aguilar, J., Hibbs, M. L., and Springer, T. A. (1994) Multinucleated giant cell formation induced by interferon-γ. Changes in the expression and distribution of the intercellular adhesion molecule-1 during macrophages fusion and multinucleated giant cell formation. *Lab. Invest.* 71, 737–744 Medline
**SIRPα is a counter-receptor for integrin Mac-1**

13. Chen, E. H., and Olson, E. N. (2005) Unveiling the mechanisms of cell–cell fusion. *Science* **308**, 369–373 [CrossRef Medline]

14. Martens, S., and McMahon, H. T. (2008) Mechanisms of membrane fusion: disparate players and common principles. *Nat. Rev. Mol. Cell Biol.* **9**, 543–556 [CrossRef Medline]

15. Saginario, C., Sterling, H., Beckers, C., Kobayashi, R., Solimena, M., Ullu, E., and Vignery, A. (1998) MFR, a putative receptor mediating the fusion of macrophages. *Mol. Cell. Biol.* **18**, 6213–6223 [CrossRef Medline]

16. Han, X., Sterling, H., Chen, Y., Saginario, C., Brown, E. J., Frazier, W. A., Lindberg, F. P., and Vignery, A. (2000) CD47, a ligand for the macrophage fusion receptor, participates in macrophage multination. *J. Biol. Chem.* **275**, 37984–37992 [CrossRef Medline]

17. Zhang, L., and Plow, E. F. (1997) Identification and reconstruction of the binding pocket within α₅β₃ for a specific and high affinity ligand, NIF. *J. Biol. Chem.* **272**, 17558–17564 [CrossRef Medline]

18. Yakubenko, V. P., Lishko, V. K., Lam, S. C., and Ugarova, T. P. (2002) A molecular basis for integrin α₅β₃ ligand binding promiscuity. *J. Biol. Chem.* **277**, 48635–48642 [CrossRef Medline]

19. Duff, J. L., Monia, B. P., and Berk, B. C. (1995) Mitogen-activated protein (MAP) kinase is regulated by the MAP kinase phosphatase (MKP-1) in vascular smooth muscle cells. *J. Biol. Chem.* **270**, 7161–7166 [CrossRef Medline]

20. Flick, M. J., Du, X., Witte, D. P., Jirousková, M., Soloviev, D. A., Busuttil, R., Cummings, R. D., and Parkos, C. A. (2010) The role of cis dimerization of signal regulatory protein α (SIRPα) in binding to CD47. *J. Biol. Chem.* **285**, 37953–37963 [CrossRef Medline]

21. Schober, J. M., Chen, N., Grzeskiewicz, T. M., Jovanovic, I., Emeson, E. E., Ugarova, T. P., Ye, R. D., Lau, L. F., and Lam, S. C. (2002) Identification of integrin α(M)β(2) as an adhesion receptor on peripheral blood monocytes for Cyr61 (CCN1) and connective tissue growth factor (CCN2): immediate-early gene products expressed in atherosclerotic lesions. *Blood* **99**, 4457–4465 [CrossRef Medline]

22. Podolnikova, N. P., Brothwell, J. A., and Ugarova, T. P. (2015) The opioid peptide dynorphin A induces leukocyte responses via integrin Mac-1 (α₅β₂, CD11b/CD18). *Mol. Pain* **11**, 33 [CrossRef Medline]

23. Lishko, V. K., Moreno, B., Podolnikova, N. P., and Ugarova, T. P. (2016) Two conformations of the integrin A-domain (I-domain): a pathway for activation? *Structure* **3**, 1333–1340 [CrossRef Medline]

24. Lee, J.-O., Rieu, P., Arnaut, M. A., and Liddington, R. (1995) Crystal structure of the A domain from the α subunit of integrin CR3 (CD11b/CD18). *Cell* **80**, 631–638 [CrossRef Medline]

25. Xiong, J.-P., Li, R., Essafi, M., Stehle, T., and Arnaout, M. A. (2000) An isoleucine-based allosteric switch controls affinity and shape shifting in integrin CD11b A-domain. *J. Biol. Chem.* **275**, 38762–38767 [CrossRef Medline]

26. Podolniková, N. P., Podolníková, A. V., Haas, T. A., Liszkó, V. K., and Ugarova, T. P. (2015) Ligand recognition specificity of leukocyte integrin αMβ2 (Mac-1, CD11b/CD18) and its functional consequences. *Biochemistry* **54**, 1408–1420 [CrossRef Medline]

27. Chiozzi, P., Sanz, J. M., Ferrari, D., Falzoni, S., Aeoliti, A., Buel, G. N., Collo, G., and Di Virgilio, F. (1997) Spontaneous cell fusion in macrophage cultures expressing high levels of the P2Z/P2X7 receptor. *J. Cell Biol.* **138**, 697–706 [CrossRef Medline]

28. Shilagardi, K., Li, S., Luo, F., Marikar, F., Duan, R., Jin, P., Kim, J. H., Murnen, K., and Chen, E. H. (2013) Actin-propelled invasive membrane protrusions promote fusogenic protein engagement during cell–cell fusion. *Science* **340**, 359–363 [CrossRef Medline]

29. Xiong, V. P., Solovjov, D. A., Zhang, L., Yee, V. C., Plow, E. F., and Ugarova, T. P. (2001) Identification of the binding site for fibrinogen recognition peptide γ383–395 within the α₅β₃-domain of integrin α₅β₃. *J. Biol. Chem.* **276**, 13995–14003 [CrossRef Medline]
50. Lishko, V. K., Yakubenko, V. P., and Ugarova, T. P. (2003) The interplay between integrins $\alpha_5\beta_1$ and $\alpha_4\beta_1$ during cell migration to fibronectin. *Exp. Cell Res.* **283**, 116–126 CrossRef Medline

51. Yalamanchili, P., Lu, C., Oxvig, C., and Springer, T. A. (2000) Folding and function of I domain-deleted Mac-1 and lymphocyte function-associated antigen-1. *J. Biol. Chem.* **275**, 21877–21882 CrossRef Medline

52. Frank, R. (1992) Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron* **48**, 9217–9232 CrossRef

53. Kramer, A., and Schneider-Mergener, J. (1998) Synthesis and screening of peptide libraries on continuous cellulose membrane supports. *Methods Mol. Biol.* **87**, 25–39 Medline