Normal Ligand Binding and Signaling by CD47 (Integrin-associated Protein) Requires a Long Range Disulfide Bond between the Extracellular and Membrane-spanning Domains*

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Robert A. Rebres, Louise E. Vaz, Jennifer M. Green‡, and Eric J. Brown§
From the Program in Microbial Pathogenesis and Host Defense and Department of Medicine, University of California, San Francisco, California 94143

CD47 is a unique member of the Ig superfamily with a single extracellular Ig domain followed by a multiply membrane-spanning (MMS) domain with five transmembrane segments, implicated in both integrin-dependent and -independent signaling cascades. Essentially all functions of CD47 require both the Ig and MMS domains, raising the possibility that interaction between the two domains is required for normal function. Conservation of Cys residues among CD47 homologues suggested the existence of a disulfide bond between the Ig and MMS domains that was confirmed by chemical digestion and mapped to Cys33 and Cys263. Subtle changes in CD47 conformation in the absence of the disulfide were suggested by decreased binding of two anti-Ig domain monoclonal antibodies, decreased SIRPα1 binding, and reduced CD47/SIRPα1-mediated cell adhesion. Mutagenesis to prevent formation of this disulfide completely disrupted CD47 signaling independent of effects on ligand binding, as assessed by T cell interleukin-2 secretion and Ca2+ responses. Loss of the disulfide did not affect membrane raft localization of CD47 or its association with αβ2 integrin. Thus, a disulfide bond between the Ig and MMS domains of CD47 is required for normal ligand binding and signal transduction.

A cell responds to its environment through cues arising from binding of soluble mediators of cell-cell communication and from interaction with insoluble molecules in the extracellular matrix (ECM)1 or on adjoining cells. A detailed understanding of how specific plasma membrane molecules transduce information from the extracellular milieu to the cytoplasm and how these are spatially and temporally integrated is required to begin to understand, predict, and potentially regulate these responses in normal and pathologic conditions. Two major models of transmembrane signal transduction in response to ligand binding have been advanced. For many Ig family and growth factor receptors, signaling is initiated by receptor clustering. Other receptors, like the heptaspanin family of heterotrimeric G protein-coupled receptors, seem to signal ligand binding through conformational changes in the membrane-spanning domain that lead ultimately to the activation of effector cascades.

CD47 is an Ig superfamily member involved in signaling from both cell-cell and cell-ECM interactions. Coligation of CD47 and the T cell antigen receptor (TCR) is a synergistic signal for activation of T lymphocytes (1–3) that requires CD47-induced association of protein kinase Cθ with cytoskeleton (3). In addition, CD47 can exist in a plasma membrane complex with the integrin αβ2, which modulates integrin function and has signal transduction properties distinct from either CD47 or the integrin in isolation (4–7). CD47 binds directly to thrombospondin, a protein of the provisional ECM at sites of inflammation (8), and to SIRPα1, a broadly expressed plasma membrane molecule most highly represented on macrophages, macrophages, and dendritic cells (9, 10). Interaction with these ligands can lead to cell adhesion to ECM (6, 7, 11), to cell-cell aggregation (12, 13), or to alterations in cell behavior via heterotrimeric G protein-dependent and -independent mechanisms (3, 6, 14). CD47-deficient mice or cells show a variety of abnormalities consistent with a significant role for this molecule in modulating cell responses to adhesive stimuli. Since CD47 is an Ig family member that can in at least some circumstances signal via heterotrimeric G proteins, the paradigm for signal transduction through this interesting molecule is uncertain. CD47 is an unusual member of the Ig superfamily because, in addition to a single Ig domain, it has a highly hydrophobic, multiply membrane-spanning (MMS) domain that is thought to contain five transmembrane segments (15). Structure-function studies have demonstrated that both the Ig domain and the MMS domain of CD47 are essential for its role in signal transduction as well as for localization of CD47 to the cholesterol-rich plasma membrane domains known as glycosphingolipid-enriched membranes (gems) or rafts (1, 2, 4). Chimeric molecules in which the CD8 Ig domain or the FLAG epitope was substituted for CD47’s Ig were mislocalized and nonfunctional, even when the new extracellular domain was ligated by appropriate antibodies (3). This requirement for its specific Ig domain is unlike those of more conventional Ig superfamily signaling molecules (16) and suggests that CD47 aggregation is insufficient to initiate signaling. Thus, the Ig domain plays a fundamental role in CD47 signal transduction in addition to its binding of the ligands thrombospondin and...
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SIRPα1, which suggests the possibility that the CD47 Ig domain is required for a signaling-compotent conformation of the molecule. The purpose of the present study was to determine the reason for the requirement for the CD47 Ig domain in its signaling function. We have found that there is a long range disulfide bond between Cys\(^{36}\) and Cys\(^{263}\) in human CD47 that is required for signal transduction as well as for normal SIRPα1 binding. Moreover, the cysteines involved in this long range disulfide are conserved in all species’ CD47 paralogs and in the poxvirus molecules of unknown function with structural homology to CD47. The unusual requirement for the CD47 Ig domain in signaling function can be explained at least in part by its interaction with the MMS domain, leading to appropriate conformation not only for ligand binding but for association with intracellular signaling cascades as well.

MATERIALS AND METHODS

Cell Culture—Jurkat cells and the CD47-deficient Jurkat clone JINB8 (2) were maintained in RPMI with 10% FBS, nonessential amino acids, 2 mM glutamine, 50 \(\mu\)M \(\beta\)-mercaptoethanol, and 50 \(\mu\)g/ml gentamicin. OV10 cells expressing human \(\beta\) integrin were maintained in Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum, 10 \(\mu\)g/ml lipofectamine, and 100 \(\mu\)g/ml hygromycin.

Antibodies and Reagents—The following mAbs were employed: anti-CD47 Ig domain 2D3, 2E1, 1F7, B6H12, 2B7, 3G3, and 4I1 (all IgG) and 10F2 (IgM) (17–19); anti-CD47 C-terminal peptide (NQQTIQP-PKRNN) mAb 131 and mAb 151 (distinct epitopes) (15); anti-FLAG epitope tag (M2; Sigma), myeloma IgG1 (MOPC-21; Sigma), anti-CD8α (33.3; Pharmingen), anti-CD28 (15E8; Caltag); anti-CD3 (OKT3); anti-CD47, and anti-CD8-MMS include a FLAG epitope tag or murine CD8 Ig domain, respectively, linked to the MMS domain, leading to appropriate conformation not only for ligand binding but for interaction with intracellular signaling cascades as well.

For evaluation of the trypsin sensitivity of the CD47 mutants, cells were pretreated with 10 mM iodoacetamide in PBS for 15 min on ice, lysed in 10 mM CHAPS, 10 mM iodoacetamide, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and washed with Iscove’s modified Dulbecco’s medium plus 0.1% fatty acid-free \(\beta\)-mercaptoethanol at 60 °C for 15 min, run on 4–20% gradient SDS-PAGE gels, blotted to polyvinylidene difluoride, and probed with mAb 151.

For the purpose of affinity isolation of JINB8 transfected CD47 was performed for endoprotease Arg-C digests. Briefly, cells were treated with 20 \(\mu\)M iodoacetamide in PBS in the dark at 0 °C for 45 min and then lysed in 10 mM CHAPS, 10 mM iodoacetamide, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride in PBS (pH 7.4) for 4 °C for 30 min. Lysates were cleared by centrifugation at 14,000 \(\times\) g for 5 min, and CD47 protein was immunoprecipitated with anti-CD47-Sepharose, washed, eluted, and dialyzed as described (17). Digests were performed in 10 mM CHAPS, 0.1 mM (pH 8.2), with 20 \(\mu\)g/ml protein sequence grading endoprotease Arg-C (Sigma) for 1 h at 37 °C. Samples were then treated with Laemmli sample buffer with or without \(\beta\)-mercaptoethanol at 60 °C for 15 min, run on 4–20% gradient SDS-PAGE gels, blotted to polyvinylidene difluoride, and probed with mAb 151.

For evaluation of the tryptic sensitivity of the CD47 mutants, cells were pretreated with 10 mM iodoacetamide in PBS for 15 min on ice, washed in 1% Triton X-100, 20 mM Tris, 140 mM NaCl, 10 mM iodoacetamide, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride in PBS (pH 7.4) for 30 min and centrifuged for 14,000 \(\times\) g for 5 min. The cleared whole cell lysates were then digested with trypsin at 2.5 mg/mL for 30 min at 37 °C, and the reaction was stopped by the addition of Laemmli sample buffer and incubation at 60 °C for 15 min. Samples were then Western blotted with anti-CD47 Ig domain-specific mAb B6H12.

SIRPα1-Fc Binding and SIRPα1/CD47-mediated Cell Aggregation Assays—Assessment of binding of SIRPα1-Fc protein, cells were incubated with SIRPα1-Fc fusion protein, anti-CD47, or control mAb in PBS with 1% bovine serum albumin for 30 min on ice, washed, and labeled with anti-human IgG-Fc-FITC or anti-mouse IgG-FITC. Samples were then analyzed on a flow cytometer, and the mean fluorescence was determined, which is the ratio of SIRPα1 mediated CD47 fluorescence was calculated. For examination of mAb 10G2 binding, cells were pretreated with 100 ng/mL phorbol 12-myristate 13-acetate plus 2 \(\mu\)M ionomycin for 18 h prior to analysis, and the percentage of positive staining was calculated. For treatment with methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD), cells were harvested in PBS with 5 mM EDTA, washed once with PBS and once with Iscove’s modified Dulbecco’s medium plus 0.1% fatty acid-free bovine serum albumin. Cells were then resuspended at 2.5 \(\times\) 10^5 cells/mL with or without 10 mM M\(\beta\)CD, incubated at 37 °C for 10–15 min, and washed with Iscove’s modified Dulbecco’s medium plus fatty acid-free bovine serum albumin. Samples were then processed for flow cytometry as described.

JINB8 cells transfected with CD47 mutants were loaded with carboxy-SNARF-1 AM (Molecular Probes, Inc., Eugene, OR), and JINB8 cells transfected with SIRPα1/CD7 or vector were loaded with Cell-Clear Green CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes). Cells expressing normal CD47 or mutants were next incubated with anti-CD47 (2D3)-coated 4.5 \(\mu\)M magnetic beads (Dynal), and 1 \(\times\) 10^5 of these cells were added to a 24-well plate well along with 1 \(\times\) 10^5 SIRPα1/CD7 cells in 1 ml of RPMI medium. After incubation for 1–8 h, cells were transferred to Eppendorf tubes, and bound-bound cells were separated with a magnet. Approximately 75% of SNARF-1-labeled

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(CD47<sup>-</sup>) cells were isolated by this procedure. Magnet-associated cells were lysed in 1% Nonidet P-40, 10 mM Tris (pH 7.4), 145 mM NaCl, and cleared lysates were evaluated in a fluorescence plate reader at SNARF-1 and CellTracker Green wavelengths versus standards of known cell number. Adhesion indices were calculated as the ratios of green (SIRP<sub>α</sub>1-expressing) to red (CD47-expressing) cells adherent to the magnet after correction for background (SIRPα1-independent adhesion) by subtracting the adhesion index for SIRPα1/CD7-deficient

FIG. 1. CD47 structure, sites of mutagenesis, expression, and amino acid sequence conservation among homologues. A, schematic cartoon showing amino acid sequence of human CD47 and the amino acid substitutions used in mutants. The Cys/Ser mutants included the C33S Ig domain mutant and CC259/263S, C259S, and C263S MMS domain mutants. Non-Cys/Ser mutants of the MMS domain included the first and second ICL (1<sup>st</sup> ICL and 2<sup>nd</sup> ICL) and third transmembrane (3<sup>rd</sup> TM) mutants as described under “Materials and Methods.” B, cytometric analysis of Cys/Ser mutant and wild type CD47 expression levels on JinB8 transfectants using mAb 2D3. Non-Cys/Ser mutants had similar expression levels (not shown). C, amino acid sequences are aligned for rat (AAB70273), mouse (S36646), human (C48997), cow (AJ245943), and pig (AAK15531) CD47, A38 protein from Vaccinia virus WR (P24763) and Variola virus (P33853), m128L protein from Myxoma Virus (NP_051842), and gp128L protein from rabbit fibroma virus (NP_052017). Standard single letter amino acid abbreviations are used. Cys residues theorized to form disulfide bonds are in boldface type, and additional conserved Cys or Ser residues are italicized.
cells (typically ~10% of the adhesion of the SIRPa1/CD7-expressing cells). A typical adhesion index for cells expressing wild-type CD47 was 0.5, which represented 1 SIRPa1/CD7+ cell pulled down per 2 CD47+ cells. All experimental points were assayed in triplicate.

**IL-2 Assays**—Human CD47-transfected murine 3.L2 T cells were stimulated by murine CH27 B cells presenting peptide antigen as described. Cells were incubated with 5 μg/ml 125I-mAb in growth medium at 4°C; washed; and lysed in 20 mM Tris-HCl, pH 8.2, 140 mM NaCl, 2 mM EDTA, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.1% Brij 58. Sucrose solution was layered over a volume of 60% sucrose. 25 and 5% sucrose layers were added to form a step gradient, which was centrifuged at 170,000 g at 4°C. Fractions of 0.5 ml were collected from the top of the gradient, and radioactivity in each fraction as well as the pellet was assessed.

**Isolation of Membrane Rafts**—The location of cell surface proteins in sucrose density gradients was evaluated using tracer 125I-labeled antibodies as described. Cells were incubated with 5 μg/ml 125I-mAb in growth medium at 4°C; washed; and lysed in 20 mM Tris-HCl, pH 8.2, 140 mM NaCl, 2 mM EDTA, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.1% Brij 58. Sucrose solution was added to a final concentration of 40% using a stock of 60% sucrose in 20 mM Tris-HCl, pH 8.2, 140 mM NaCl, 2 mM EDTA, and this mixture was layered over a volume of 60% sucrose. 25 and 5% sucrose layers were added to form a step gradient, which was centrifuged at 170,000 g for 18 h at 4°C. Fractions of 0.5 ml were collected from the top of the gradient, and radioactivity in each fraction as well as the pellet was assessed.

**Measurement of Ca2+ Flux following Receptor Cross-linking**—Jurkat or JIN8 cells at 2 × 106 cells/ml in RPMI complete medium were incubated with 5 μM fura-2-AM at 37°C for 20 min and then diluted 10-fold and incubated an additional 20 min. Cells were then washed and incubated with the indicated concentrations of mAb on ice for 20 min. After labeling, cells were washed once with RPMI medium and twice with calcium buffer (25 mM Hepes, 125 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 0.5 mM MgCl2, 1 mM CaCl2, pH 7.4) and resuspended at 2.5 × 106 cells/ml in calcium buffer on ice. Fluorescence changes of a 2-ml stirred cell suspension warmed to 37°C were monitored with a F-2000 or F-4500 spectrophotometer (Hitachi Instruments, Danbury, CT) using 340- and 380-nm excitation wavelengths and 510-nm emission wavelength following the addition of 10 μg/ml secondary antibody. Calcium concentrations were calculated as described by Grynkiewicz et al. (22). Calcium flux from CD47 cross-linking was identical when cells were coated with 1 or 10 μg/ml anti-CD47 mAb, but no flux occurred when 0.1 μg/ml anti-CD47 was used or when the cross-linking antibody was omitted, confirming that CD47 aggregation was required for the rise in [Ca2+]i. The addition of 2 μM ionomycin served as a positive control for Ca2+ flux.

**Isolation of αβ3 Integrin-containing Protein Complexes**—Complexes were isolated as described (4). Briefly, OV10 cells expressing CD47 or mutants were mixed slowly in HBSS with anti-β3 integrin-coated magnetic beads for 15 min at 37°C. Adherent cells were separated with a magnet and lysed in CHAPS buffer with mixing for 10 min. Beads were resolated, bead-associated protein complexes were eluted with Laemmli sample buffer, and β3-associated CD47 was quantitated as described (4). Values are expressed in relative units based on densitometry of chemiluminescence-exposed bands on x-ray films.

**Statistical Analysis**—All experiments were repeated at least three times. Error bars in graphs depict S.E. The statistical significance of each set of results was evaluated by performing a one-way analysis of variance followed by Dunnett or individual t tests as appropriate. A p value of <0.05 was considered significant.

**FIG. 2.** A long range disulfide bond between the Ig and MMS domains of CD47. A, schematic diagram of CD47 showing the location of tryptophan residues and composition and molecular weight of theorized BNPS cleavage products in the presence or absence of the potential disulfide bond. B, CD47 was isolated from human placenta and digested with BNPS-skatole as described. The resulting peptides were resolved on Tris-Tricine gels and detected by Western blotting using mAb 131 specific for the CD47 cytoplasmic tail. C, schematic diagram of CD47 showing the location of arginine residues and composition and molecular weight of theorized endoproteinase (Endo) Arg-C cleavage products in the presence or absence of the potential disulfide bond. D, CD47 was isolated from placenta or Jurkat transfectants and digested with endoproteinase Arg-C. The resulting peptides were resolved by SDS-PAGE and Western blotting using mAb 151 specific for the CD47 cytoplasmic tail.
RESULTS

A Long Range Disulfide Bond Exists between the Ig and MMS Domains of CD47—Previous studies have indicated a requirement for both Ig and MMS domains of CD47 to mediate virtually all described functions of CD47 and for its efficient localization to membrane rafts. This suggests that an interaction between these domains may be important for function and subcellular localization. The Ig domain contains conserved cysteine residues at positions 41 and 114 that are necessary for the Ig domain formation as well as a potentially free Cys in the amino terminus of the protein in the absence of reduction, within 5 kDa of the C terminus yet is covalently linked to the COOH terminus of CD47. Previous studies have indicated a cysteine carboxyl-terminal to this cleavage site (Met172) would have yielded a significantly larger fragment (14 kDa) and is therefore unlikely to account for this small fragment. Whatever the identity of the cleavage site, it is within 5 kDa of the C terminus yet is covalently linked to the amino terminus of the protein in the absence of reduction, demonstrating that a cysteine carboxyl-terminal to this cleavage site is involved in a disulfide bond.

CD47 point mutants C33S, C259S, and C263S were transfected into the CD47-deficient Jurkat clone JInB8 (2) to identify the location of the cysteines involved in disulfide bonding. Unfortunately, it proved impossible to obtain sufficient quantities of mutant CD47 for BNPS digestion analysis. Digestion with endoproteinase Arg-C, however, yielded sufficient material for analysis. Theoretical sites of cleavage are shown in Fig. 2C. While the anticipated ~14-kDa fragment was observed in nearly 100% yield with reduction of digested placental CD47, nonreduced fragments reactive with the carboxyl-terminal mAb migrated at ~50 kDa, similar to undigested CD47 (Fig. 2D). Thus, all of the C-terminal fragments remained linked to additional fragments under nonreducing conditions. Analysis of Jurkat-expressed wild type CD47 showed that digestion yielded >95% release of a 14-kDa fragment with reduction, but ~75% of the 14-kDa fragment was retained in an ~50-kDa band under nonreducing conditions. Digestion of the C259S mutant CD47 did not increase the amount of free 14-kDa fragment without reduction. In contrast, digestion of both the C33S and C263S mutants led to quantitative release of the 14-kDa carboxyl-terminal fragment without reduction. Thus, Cys33 and Cys263 of CD47 are required for disulfide-dependent retention of the 14-kDa band with other fragments after Arg-C digestion. These data demonstrate that there is a disulfide bond between Cys33 and Cys263 in CD47 isolated from Jurkat T cells, and a similar disulfide between Ig and MMS domains is present in placental CD47.

Cys33 and Cys263, but not Cys259, are conserved in all members of the CD47 family, including several poxvirus proteins (Fig. 1C). Based on conservation of these Cys residues, this long range disulfide bond is probably preserved in all members of the CD47 family.

Trypsin Digestion of CD47 Mutants—Whole cell lysates were digested with trypsin to examine differences in protease sensitivity of wild type or mutant CD47 molecules. Total cellular protein was >95% digested as determined by loss of Coomassie Blue staining of the digested lysate (data not shown). The digested lysate was separated by SDS-PAGE, and remaining CD47 was detected using an anti-Ig domain mAb, B6H12. Wild type CD47 was resistant to trypsin digestion, while a molecule in which the MMS domain was replaced by the CD7 transmembrane domain (CD7/CD7) was sensitive, suggesting that the presence of the CD47 MMS domain decreased trypsin sensitivity of the B6H12 epitope in the Ig domain. Like wild type CD47 (17), CD47/CD7 can form multimers on SDS-PAGE, and both monomeric and dimeric forms were detected. Although the

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**Table:**

| Trypsin: | + | - | - | - | - | - | + |
|---|---|---|---|---|---|---|---|
| MW | 160 | 150 | 75 | 50 | 35 | 30 | 15 |

**Fig. 2.** Loss of Cys33 or Cys263 results in an increase in protease sensitivity of CD47. Whole cell lysates were prepared from JInB8 cells expressing wild type CD47 or C33S, C259S, or C263S mutants and were digested with trypsin for 30 min as described under “Materials and Methods.” Western blotting was performed using anti-Ig domain mAb B6H12 on nonreduced samples. The absence of the CD47 transmembrane domain or loss of the long range disulfide bond confers trypsin sensitivity to the Ig domain.

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**Fig. 3.** Long Range Disulfide Bond Exists between the Ig and MMS Domains of CD47. Whole cell lysates were prepared from JinB8 cells expressing wild type CD47 or C33S, C259S, or C263S mutants and were digested with trypsin for 30 min as described under “Materials and Methods.” Western blotting was performed using anti-Ig domain mAb B6H12 on nonreduced samples. The absence of the CD47 transmembrane domain or loss of the long range disulfide bond confers trypsin sensitivity to the Ig domain.
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### Table I

| mAb staining intensity | 1F7 | 2B7 | B6H12 | 2D3 | 2E11 | 3G3 | 430 | 10G2 |
|------------------------|-----|-----|-------|-----|------|-----|-----|------|
| CD47                   | 72.9| 67.9| 46.0  | 84.5| 78.4 | 83.4| 100 | 74.4 ± 3.9 |
| CD47/CD7               | 74.1| 65.9| 40.2  | 91.8| 78.3 | 80.3| 100 | 4.8 ± 2.0 |
| CD47/GPI               | 67.2| 57.4| 43.3  | 87.9| 66.9 | 76.7| 100 | 4.9 ± 2.0 |
| 1st ICL                | 74.9| 65.8| 42.2  | 78.8| 74.9 | 88.2| 100 | 37.2 ± 7.2 |
| 2nd ICL                | 70.0| 64.8| 43.2  | 80.4| 75.2 | 81.1| 100 | 37.2 ± 7.2 |
| 3rd TM                 | 73.5| 67.2| 46.4  | 76.6| 76.6 | 84.9| 100 | 64.1 ± 11.7 |
| C259/263S              | 68.4| 58.4| 34.1  | 76.0| 67.2 | 73.6| 100 | 16.8 ± 3.3 |
| C33S                   | 66.4| 58.9| 24.0  | 75.7| 68.1 | 78.6| 100 | 17.1 ± 6.3 |
| CD47/CD7               | 72.9| 67.9| 46.0  | 84.5| 78.4 | 83.4| 100 | 74.4 ± 3.9 |
| CD47/CD7               | 74.1| 65.9| 40.2  | 91.8| 78.3 | 80.3| 100 | 4.8 ± 2.0 |
| CD47/GPI               | 67.2| 57.4| 43.3  | 87.9| 66.9 | 76.7| 100 | 4.9 ± 2.0 |
| 1st ICL                | 74.9| 65.8| 42.2  | 78.8| 74.9 | 88.2| 100 | 37.2 ± 7.2 |
| 2nd ICL                | 70.0| 64.8| 43.2  | 80.4| 75.2 | 81.1| 100 | 37.2 ± 7.2 |
| 3rd TM                 | 73.5| 67.2| 46.4  | 76.6| 76.6 | 84.9| 100 | 64.1 ± 11.7 |
| C259/263S              | 68.4| 58.4| 34.1  | 76.0| 67.2 | 73.6| 100 | 16.8 ± 3.3 |
| C33S                   | 66.4| 58.9| 24.0  | 75.7| 68.1 | 78.6| 100 | 17.1 ± 6.3 |

* Cells were labeled by standard methods on ice with the indicated mAb and FITC-conjugated secondary antibody. Mean fluorescence intensity was standardized to that of mAb 430, set to equal 100 units. Results shown are from a representative experiment.

**For maximal induction of 10G2 epitope expression, cells were treated with 100 ng/ml phorbol 12-myristate 13-acetate plus 2 μM ionomycin for 18 h. These values are reported as percentage positive (mean ± S.E., n = 3 experiments).**

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C259S mutant showed trypsin resistance similar to wild type CD47, the C33S and C263S mutants showed much greater trypsin sensitivity (Fig. 3), suggesting increased accessibility of trypsin cleavage sites in the Ig domain in the absence of a disulfide between these two cysteines. Thus, loss of the long range disulfide increases the susceptibility the CD47 Ig domain to trypsin digestion.

In order to further evaluate the effects of these Cys/Ser point mutations on the accessibility of Ig domain epitopes under more physiologic conditions, binding of a panel of mAbs that recognize the CD47 Ig domain was assessed by flow cytometry. All of the antibodies shown in Table I demonstrated clear binding to each of the point mutants, suggesting that all of the mutants have essentially normally folded Ig domains. There was a slight decrease in epitope expression or accessibility for two of the eight mAbs, B6H12 and 10G2, to the Ig and MMS Cys/Ser mutants, although several mAbs with overlapping epitopes (as indicated by competitive binding and Ig domain point mutant studies) bound normally to these same mutants. The mAb B6H12 binds a temperature-sensitive epitope (23) and is inhibitory in integrin-dependent assays of CD47 function (5–8). The mAb 10G2 recognizes a site that contributes to soluble CD23 binding by the CD47-α,β,γ complex (19). Expression of the 10G2 epitope is influenced by interaction of the MMS domain with cholesterol (4). Thus, loss of the Cys33–Cys263 disulfide slightly alters mAb recognition and markedly increases trypsin sensitivity of the Ig domain, consistent with a conformational change in CD47 in the absence of the long range disulfide.

Loss of the Cys33–Cys263 Disulfide Affects SIRPα1 Ligand Binding and CD47-SIRPα1-mediated Cell Aggregation—The likelihood that there were subtle conformational effects from the loss of the Cys33–Cys263 disulfide raised the possibility that loss of the disulfide might affect ligand binding and/or cell-cell interaction. To determine the importance of the disulfide bond in binding of SIRPα1, a ligand for CD47 expressed prominently on macrophages and dendritic cells, the binding of soluble dimeric SIRPα1 was assessed. When binding was assessed at a SIRPα1-Fc concentration within the linear range of the binding curve, there was an ~50% reduction in binding of SIRPα1-Fc to CD47 mutants that could not form the long range disulfide (Fig. 4A). The single point mutant C263S showed as great a defect in ligand binding as the double CC259/263SS mutant, and the C259S showed no defect in ligand binding. The absence of the MMS altogether in the CD47/CD7 chimera (5) showed a similar reduction in ligand binding, indicating that the principal contribution of the MMS to ligand binding is the disulfide between Cys263 and Cys33. The CC259/263SS mutation also reduced SIRPα1 binding in an unrelated cell type, the ovarian carcinoma OV-10 (Fig. 4A, right). Binding of SIRPα1-Fc was clearly reduced in the Cys/Ser mutants over a broad concentration range (Fig. 4B).

To determine whether this loss of ligand binding had functional consequences, CD47-SIRPα1-mediated cell-cell adhesion was evaluated (Fig. 5). Compared with JinB8 cells expressing wild type CD47, cells transfected with the CD47/CD7 chimera adhered to SIRPα1-expressing cells less well, demonstrating...

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**E. J. Brown, unpublished data.**
that the MMS domain is required for optimal CD47-mediated cell aggregation. The CD47 point mutants lacking the long range disulfide showed as large a defect in adhesion as the CD47/CD7 chimera, suggesting that the disulfide is a critical component of the contribution of the MMS domain to cell-cell adhesion. In this assay, similar to soluble SIRPα1/H9251 binding, the C259S mutant was normal, supporting the conclusion that Cys263 is the relevant MMS domain amino acid for the disulfide bond. Other MMS domain mutants showed no defect in cell-cell adhesion, supporting the specificity of the requirement for the Cys33–Cys263 disulfide for optimal SIRPα1 binding (Fig. 5, data not shown).

**Loss of the Cys33–Cys263 Disulfide Affects CD47 Synergy with TCR**—While loss of the long range disulfide bond reduced ligand binding activity, it was unclear if the signaling activity of CD47 was similarly affected. To determine the role for the disulfide bond in CD47 signaling, functional assays using mAb that bound wild type and mutant CD47 identically were employed. Murine 3.L2 hybridomas (21) or CD47-deficient JinB8 cells expressing CD47 molecules containing point mutations that prevent disulfide formation were compared with cells expressing wild type CD47 or several other mutations in the MMS domain (Fig. 1). All CD47 mutants were expressed at levels comparable with or greater than wild type CD47 as assessed by flow cytometry or Western blotting (Fig. 1B). Several studies have demonstrated CD47 synergy with TCR ligation in T cell activation leading to IL2 synthesis (1, 2). 3.L2 cells transfected with normal human CD47 show an augmented response to antigenic peptides when anti-CD47 is allowed to bind to Fcγ receptors on the antigen-presenting cells (1) (Fig. 6A). Antigen-specific murine 3.L2 T cell transfectants were incubated with CH27 B cell transfectants in the presence or absence of antigen for 20 h, and supernatant IL-2 content was determined by enzyme-linked immunosorbent assay. A, antigen dose responses for 3.L2 cells expressing wild type CD47 incubated with 2D3, B6H12, IB4, and MOPC antibodies. Similar assessments of synergy for first ICL (B), C33S (C), and C259/263S (D) mutants are shown from representative experiments. Multiple independent transfections of each mutant were evaluated with similar results. Second ICL and third transmembrane mutants showed effective synergy (not shown).
CD47-induced Ca\(^{2+}\) response is deficient in Cys/Ser mutants. Fura-2-loaded Jurkat cells transfected with FLAG-MMS or CD8-MMS (A) or JinB8 cells transfected with CD47, CD47/CD7, or CD47/GPI (B) were coated with the indicated mAbs at 4 °C and warmed to 37 °C for 3 min prior to treatment with 10 μg/ml goat anti-mouse IgG. Intracytoplasmic Ca\(^{2+}\) concentration was calculated from fluorescence ratios during a 10-min recording. C, a summary of responses showing integrated [Ca\(^{2+}\)](i) flux was determined for CD47 (WT), control transfectants (Vector) and various CD47 mutants, as indicated. All cells showed similar CD3 responses with ~500 nm peak responses. Stably transfected cells from two independent transfections were analyzed for the C259S/C263S MMS mutant (CC/SS#1 and CC/SS#2) and for the C33S Ig mutant (C33S#1 and C33S#2). *, p < 0.01 versus wild type CD47. D, JinB8 cells transfected with C259S CD47 showed a normal increase in [Ca\(^{2+}\)](i) (compare CD47; panel B), while the C263S transfecant was unable to signal [Ca\(^{2+}\)](i) flux.

Changes in membrane raft localization of Cys/Ser CD47 Are Not Sufficient to Explain Loss of Function—Membrane raft localization is thought to be necessary for CD47 function (3). Therefore, we evaluated if loss of the disulfide bond affected raft localization of CD47. Sucrose gradient fractionation studies showed a modest decrease in raft localization of Cys/Ser mutants relative to wild-type CD47 (Fig. 8A). A similar modest decrease was observed for the second ICL mutant that retains completely normal function in all assays, indicating that this extent of alteration does not account for signaling dysfunction of Cys/Ser mutants.

**Fig. 8.** Altered raft distribution of CD47 mutants does not account for signaling dysfunction of Cys/Ser mutants. A, extent of concentration of CD47 mutants in membrane rafts compared with wild type CD47 was determined as described under “Materials and Methods.” Data are summary of three independent experiments. *, p < 0.05 versus normal CD47. B, effect of MβCD on SIRPα1-Fc binding. Jurkat or OV10 cells were treated with 10 mM MβCD for 15 min at 37 °C, and SIRPα1-Fc or mAb binding was assessed as described. mAbs evaluated included anti-CD47 mAbs (2D3 and 2B7), anti-integrin β\(_{1}\), anti-integrin β\(_{3}\), and anti-HLA. Data show staining by each mAb or SIRPα1-Fc binding.
The disulfide-linked MMS domain is sufficient for stable interaction with αβ3 integrin. Association of CD47 with αβ3 was determined as described under “Materials and Methods.” The amount of CD47 co-precipitated with αβ3 is depicted, with the extent of association of wild type CD47 set to equal 1 unit. Data are a summary of three independent experiments.

Interdomain disulfide formation in the CD47 MMS domain is critical to modulate Ig domain conformation, as previously described (4). While the absence of the MMS domain prevented formation of a stable protein complex (Fig. 9A), the absence of the disulfide bond did not inhibit stable interaction with CD47 association with αβ3 integrin. The amount of CD47 co-precipitated with αβ3 was significantly decreased by MβCD, suggesting that an inability to associate with cholesterol was not the reason for reduced SIRPα1 binding by these mutants. The fact that one mAb (B6H12) blocks CD47-SIRPα1 interaction, but it is of interest that several other mAbs that block SIRPα1 interaction with CD47 bind equivalently to wild type CD47 and the point mutants lacking the disulfide bond. The fact that one mAb showing diminished binding (10G2) does not affect SIRPα1 interaction demonstrates that the ligand binding site is not the only aspect of the Ig domain affected by the constraints imposed by the long range disulfide.

Formation of CD47/αβ3 integrin complexes is not inhibited by the absence of the disulfide bond—CD47 association with αβ3 or other integrins can be important in some of its signaling functions (6, 7, 11, 15, 17, 25). αβ3 integrin association of CD47 mutants was assessed in OV10 transfectants by coprecipitation, as previously described (4). While the absence of the MMS domain prevented formation of a stable protein complex (Fig. 9A), the absence of the disulfide bond did not inhibit stable CD47 association with αβ3 integrin. Thus, the presence of the non-disulfide-linked MMS domain is sufficient for stable interaction of CD47 with αβ3.

**DISCUSSION**

The dual requirement for Ig and MMS domains of CD47 to mediate its functions and to localize to membrane rafts suggested that an interdomain interaction could be critical to CD47’s role in cell biology. The presence of cysteines in the extracellular portions of the Ig and MMS domains indicated that formation of a disulfide bond between these domains was possible. Such a covalent link could significantly constrain CD47 structure with potential consequences for ligand binding and signaling. Previous protease digestion of erythrocyte-derived CD47 by Mawby et al. (26) suggested the possible occurrence of a disulfide bond between the MMS and Ig domains. However, these studies suggested the existence of the disulfide only in a fraction of the isolated CD47, and neither the site nor the consequences of the putative interaction was defined. We now have shown that the disulfide occurs between Cys33 and Cys263 of human CD47 in Jurkat T cells. Although failure to recover relevant peptides in the absence of reduction precluded mass spectrometric proof that the disulfide between Ig and MMS domains in placental CD47 is identical, this is the most likely possibility. CD47 with this disulfide is the only detectable form in placenta. Like erythrocytes, Jurkat T cells have a small amount of CD47 apparently lacking this disulfide; whether this means that there is some cellular regulation of disulfide formation is not yet known. While Cys259 is conserved in mammalian CD47 molecules, poxvirus homologues have a Ser at this position. In contrast, all members of this protein family conserve both the Ig domain Cys and the MMS domain Cys involved in the long range disulfide (Fig. 1A). While little is known of the function of the poxvirus CD47 homologues, conservation of the relevant Cys residues suggests that the disulfide and the resulting conformational constraints are critical for function.

Consistent with this hypothesis, CD47 mutants lacking the long range disulfide were defective for multiple known functions of the molecule. Binding of CD47’s cell ligand, SIRPα1, was diminished, demonstrating that the long range disulfide constrains CD47 into a conformation optimal for ligand binding. Since SIRPα1 binds to the Ig domain of CD47 (10, 27), the additional disulfide most likely optimizes the presentation of the SIRPα1-binding face of CD47. Consistent with this mode of action, loss of the long range disulfide modestly diminished the binding of two mAbs to the CD47 Ig domain. One of those two mAbs (B6H12) blocks CD47-SIRPα1 interaction, but it is of interest that several other mAbs that block SIRPα1 interaction with CD47 bind equivalently to wild type CD47 and the point mutants lacking the disulfide bond. The fact that one mAb showing diminished binding (10G2) does not affect SIRPα1 interaction demonstrates that the ligand binding site is not the only aspect of the Ig domain affected by the constraints imposed by the long range disulfide.

Signal transduction by CD47 also depends on the long range disulfide, independent of ligand binding. The impairment of signaling by Cys/Ser mutations of CD47 was demonstrated in T cell assays in which the CD47 Ig domain was ligated by mAbs that bind equally to the Cys/Ser mutants and wild type CD47. This suggests that the Cys33–Cys263 disulfide affects not only the presentation of Ig domain epitopes to ligand but the interaction of the MMS domain with potential cytosolic signaling cascades as well. Ligation of CD47 can induce both actin polymerization and protein kinase C pathways in T cells (3); while the precise molecular mechanisms for these effects are currently unknown, failure of CD47 synergy in the absence of the long range disulfide strongly suggests that it is required for CD47-induced activation of these cascades. In this regard, it is noteworthy that major mutations in two intracytoplasmic loops and in charged or polar residues in the MMS transmembrane segments had little effect on CD47 function as we have been able to test it. Thus, the molecular mechanisms by which CD47 links to signaling cascades remain undiscovered.

These data are consistent with a model for CD47 structure and function in which the Ig and MMS domains act independently. The restrictive effect of long range disulfide linkage between Cys33 and Cys263 would place a face of the Ig domain in close apposition to the MMS domain and could lead to further noncovalent interactions between the domains. Loss of the disulfide does not disrupt raft localization of the molecule, the cholesterol dependence of ligand binding, or interaction with αβ3 integrin. Since these functions also depend on both the Ig and MMS domains, there may be interdomain interac-
tions that form independent of the Cys$^{253}$–Cys$^{263}$ bond. Clearly, disulfide-mediated interaction between the domains apparently is required for CD47 signaling after ligand binding. It is possible to envision a mechanism by which ligand-induced changes in the extracellular domain of CD47 are transmitted to intracytoplasmic enzymatic cascades via this covalent interaction between Ig and MMS domains. Additionally, this orientation of the Ig domain could influence protein/protein or protein/lipid interactions by CD47 within the plasma membrane.

Long range disulfide bonds have been identified in several members of the G-protein-coupled receptor family of heptaspanins. These include the angiotensin receptor AT1, ATP receptor P2Y1, Duffy antigen receptor, Kaposi’s sarcoma-associated herpesvirus GPCR, and several chemokine receptors (CXCR1, CCR2, and CCR5) (28–34). Like CD47, these GPCRs have a disulfide between their extracellular amino termini and the final extracellular loops in their MMS domains. Studies of these disulfides indicate that they often contribute to both ligand binding and signal transduction, presumably via conformational constraints on the MMS domain. It is intriguing that CD47 not only has a disulfide bond reminiscent of some GPCR family members but in addition can sometimes interact with heterotrimeric G proteins. Recent studies have shown that mutants of CCR5 and CXCR4 lacking the first two membrane-spanning segments retain ligand binding and signaling function, indicating that five transmembrane segments (as present in CD47) are sufficient for G-protein coupling (35). Perhaps CD47 shares an evolutionary origin with this family of signaling receptors. Although a member of the long N-terminal extracellular region family-B GPCRs (36) with Ig domains at its N terminus has been described (37), the Ig domains are not disulfide-bonded to the MMS domain.

In summary, a long range disulfide bond between the Ig domain and the MMS domain of CD47 is required for optimal ligand binding and at least some important aspects of CD47 signal transduction, and it is conserved in the CD47 family. We hypothesize that interactions between the Ig and MMS domain created by the constraints induced by this disulfide bond are critical for the function of these membrane receptors for cell-matrix and cell-cell interactions.

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Normal Ligand Binding and Signaling by CD47 (Integrin-associated Protein) Requires a Long Range Disulfide Bond between the Extracellular and Membrane-spanning Domains

Robert A. Rebres, Louise E. Vaz, Jennifer M. Green and Eric J. Brown

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