Membrane Raft Association of CD47 Is Necessary for Actin Polymerization and Protein Kinase C θ Translocation in Its Synergistic Activation of T Cells*

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CD47 is a ubiquitously expressed membrane protein with an extracellular Ig domain and a multiple membrane-spanning domain that can synergize with antigen to induce interleukin (IL)-2 secretion by T lymphocytes. Ligation of CD47 induced actin polymerization and increased protein kinase C θ (PKCθ) association with the cytoskeleton independent of antigen receptor ligation, but ligation of mutant forms of the molecule missing either the Ig domain or the multiple membrane-spanning domain did not. Simultaneous ligation of CD47 and CD3 led to additive effects on F-actin and synergistic effects on PKCθ cytoskeletal association. Disruption of membrane rafts by removal of cholesterol with cyclodextrin blocked CD47-induced actin polymerization, and mutant forms of CD47 that localized poorly to rafts failed to effect cytoskeletal rearrangement. However, raft association alone was not sufficient, because a raft-localized CD47 Ig domain bound to the membrane by a glycan phosphoinositol anchor was unable to induce actin polymerization. A mutant form of CD47 without its Ig domain that did not induce actin polymerization or localize to rafts still enhanced T cell receptor (TCR)-dependent translocation of PLCγ signaling but did not augment IL-2 secretion. Thus, CD47 synergy with TCR to increase [Ca2+]i is independent of actin and rafts but is insufficient to explain CD47 cooperation with TCR in IL-2 synthesis. Full synergy with TCR requires CD47 localization to membrane rafts where ligation leads to TCR-independent signals causing actin polymerization and PKCθ translocation.

CD47 (also known as integrin-associated protein) is a 50-kDa plasma membrane protein that was purified originally by co-immunoprecipitation with β2 integrins from placenta. Molecular cloning of CD47 cDNA revealed that it is an unusual Ig family member, with an Ig variable-like extracellular domain, a domain with five putative membrane spanning segments (MMS domain) and a short cytoplasmic tail (1). Antibodies against CD47 modulate several β2 integrin functions, such as adhesion, chemotaxis, and phagocytosis in neutrophils (2–4), and CD47-deficient animals have a defect in host defense that results from lack of phagocyte activation at the site of infection (5). In addition, CD47 can synergize with antigen to activate thymocytes and T cells, a function that appears to be integrin-independent (6, 7). Although the potential significance of this synergy is suggested by the decreased number of circulating T cells in CD47-deficient mice (5), its molecular mechanism is unknown. Cross-linking CD47 on T cells can induce a TCR-dependent rise in [Ca2+]i (8), and synergy between CD47 and TCR ligation is found in early events in T cell activation, such as ζ chain phosphorylation and ZAP-70 activation. These data suggest the possibility that CD47 cooperation with TCR in these immediate consequences of TCR ligation accounts for its synergistic effect (6), in contrast to costimulation by CD28, which cooperates at later steps in the signaling cascade leading to IL-2 synthesis (9–11). These and other differences between CD28 and CD47 (6, 12) suggest that these two cell surface molecules may cooperate with TCR by quite distinct pathways. Indeed, it has been proposed that the major effect of CD47 ligation may be simply to more efficiently present antigens to TCR.

Cytoskeletal components play critical roles in modulating T cell-APC contact, early events of TCR activation, and assembly of signaling complexes important to subsequent phenotypic responses (reviewed in Refs. 13–15). Rapid cortical actin polymerization and reorganization occurs with TCR ligation, and disruption of actin cytoskeletal dynamics prevents effective T cell activation (16, 17). Cytoskeletal rearrangements are known to be important in costimulatory CD28 and LFA-1 receptor movements during cell activation and formation of supramolecular activation clusters or immunological synapses (13, 14, 18). Moreover, TCR-stimulated alterations in the function of Vav, Rac, Cdc42, and WASP can influence cytoskeletal architecture suggesting bidirectional “cross-talk” between the cytoskeleton and TCR-initiated signaling cascades.

Recently, membrane rafts, specialized domains in the plasma membrane enriched in cholesterol and sphingolipids, have been implicated in T cell activation. During activation, TCR transiently associates with membrane rafts, which are enriched in critical signaling components such as Src family kinases, LAT, and G proteins. CD28 and several raft-associated

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1 The abbreviations used are: MMS, multiple membrane-spanning; TCR, T cell receptor; PLCγ, phospholipase Cγ; JNK, Jun N-terminal kinase; IL, interleukin; PKCθ, protein kinase Cθ; GPI, glycan phosphoinositol; Ab, antibody; mAb, monoclonal antibody; BSA, bovine serum albumin; wt, wild type.
costimulatory molecules enhance association of TCR with rafts during coligation (19–21). Disruption of raft integrity by a variety of methods inhibits early activation events following immunoreceptor ligation (22–25), supporting a critical role for these domains in signaling. Multiple mechanisms for modulation of cortical actin appear linked to rafts (26, 27), raising the possibility that raft-dependent effects on actin may be critical for T cell activation.

The recent recognition that ligation of CD47 stimulates T cell spreading (28) and that the structural requirements for spreading are identical to those for synergies with the TCR to produce IL-2 suggested that these two events may reflect signaling from CD47 to the cytoskeleton important for T cell activation. Now we have shown that CD47 ligation on surfaces stimulates an increase in F-actin and has a similar structure activity profile, and CD47 enhancement of cell spreading and actin polymerization require its localization to membrane rafts. Moreover, CD47 ligation induces association of PKCθ with the cytoskeleton, and CD47 synergizes with TCR for translocation of this important component of signaling for T cell activation. In contrast, CD47 participation in PLCγ and Ca2+ signaling is not dependent on its raft localization or its ability to induce actin polymerization and cell spreading. Thus, the effects of CD47 on tyrosine phosphorylation and Ca2+ are distinct from its effects on PKCθ and not sufficient to explain its synergy with TCR in T cell activation. Our data lead to the conclusion that there are two distinct effects of CD47 in T cell signaling. Raft association and actin polymerization are not required for CD47 synergy in the early activation events of TCR signaling such as increase in [Ca2+]i, but are required for synergy in induction of PKCθ translocation, JNK activation, and IL-2 synthesis. We propose that CD47 can interact with signaling cascades both in and out of membrane rafts and that signaling within rafts is required for its synergy with TCR in T cell activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Jurkat (E6 clone), J.RT3-T3.5 (TCR-deficient) (29), and JinB8 (CD47-deficient) cells were maintained in culture in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 50 μg/mL mercaptoethanol, and 0.1% gentamicin. Transfected Jurkat or JinB8 cells (6, 12) were maintained in the same medium in the presence of 1–2 μg/mL Genetecin (Life Technologies, Inc.). cDNA constructs and transfections were described previously (6). The transfecants used in these studies included normal form 2 CD47 (1), the CD47 Ig domain fused to the CD7 transmembrane domain (CD47-CD7), the CD47 Ig domain with a GPI addition signal (CD47-GPI), a molecule in which the CD47 Ig domain was replaced with the CD7 transmembrane domain (CD47-CD7), the CD47 Ig domain fused to the CD7 transmembrane domain (CD47-CD7), the CD47 Ig domain with a GPI addition signal (CD47-GPI), a molecule in which the CD47 Ig domain was replaced with an 8-amino acid epitope tag, FLAG (30) (FLAG-MMS), or the murine CD8 extracellular Ig domain (CD8-MMS) and have been described previously (6, 12). Expression levels of the transfected constructs in Jurkat or JinB8 cells were equivalent (6, 12). Endogenous expression levels of CD3, CD4, LFA-1, or HLA class I in the Jurkat and JinB8 cells used have been described previously (2).

**Abs and Reagents**—The following mAbs were used in this study: 2E11, D23, B6H12 (IgG1, murine anti-huCD47; Refs. 3 and 4); W6/32 (IgG1, murine anti-HLA; Ref. 31); IB4 (IgG1, murine anti-huCD8; Ref. 32); 15E8 (IgG1, murine anti-huCD28; Caltag, Burlingame, CA); 3D9 (IgG1, mouse anti-FLAG epitope; Sigma), YTS105.18 (IgG2a, rat anti-mCD8; Serotec, Raleigh, NC); OKT3 (IgG2a, murine anti-huCD3; American Type Culture Collection, Manassas, VA); anti-PLCγ1 (rabbit antisemur; Upstate Biotechnology, Inc., Lake Placid, NY). Cytochalasin D was purchased from Molecular Probes (Eugene, OR). Anti-mouse IgG-Fc fusion protein or hIgG (as control) were incubated at 1 μg/mL in medium with 10% fetal bovine serum in wells precoated with anti-human Fc. In some cases the final mAb was omitted as a control. SIRPα1-Fc fusion protein or hIgG (as control) were incubated at 1 μg/mL in medium with 10% fetal bovine serum in wells precoated with anti-human Fc.

**Disruption of Membrane Rafts with Methyl-β-cycloexetrin**—Cells were suspended at 2.5×106/ml in RPMI with 0.1% fatty acid-free BSA and 10–100 μM methyl-β-cycloexetrin for 10 min at 37°C and then washed. For reconstitution of membrane cholesterol content, cells were subsequently incubated with 1.33 mg/ml cholesterol-methyl-β-cycloexetrin in RPMI with 0.1% fatty acid-free BSA and 10–100 μM methyl-β-cycloexetrin for 10 min at 37°C and then washed. Cells were then applied to prepared 96-well plates for assessment of spreading activity and F-actin content.

**Quantitation of F-actin**—For quantitation of changes in F-actin content, 1×105 cells in RPMI with 0.1% BSA were added to 96-well plates and then fixed by addition of an equal volume of 7.4% formaldehyde at 0°C. After 30 min at 4°C, supernatants were removed, and wells were refilled with 50 μL of staining solution including of Alexa-594 phalloidin and 2 μg/ml Hoechst 33258. After 20 min at 25°C, wells were washed three times with phosphate-buffered saline and read on a Molecular Devices fMax fluorescence microplate reader at dual ex/em wavelengths for Alexa-594 and Hoechst. Average cell F-actin content was determined by comparing phallolidin and DNA staining in individual wells. Standard curves of cell number versus Hoechst or Alexa-594 phallolidin staining prepared from serial dilutions of cells added to poly-l-lysine-coated plates showed linear, specific staining over at least a 20-fold range from 5×103 to 1×106 cells/well. Results were expressed as phallolidin fluorescence/Hoechst fluorescence. All measurements were performed in quadruplicate wells.

**Isolation of Membrane Rafts**—The location of cell surface proteins in sucrose density gradients was evaluated by Western blotting of gradient fractions or by using tracer 125I-labeled antibodies as described (28). In tracer studies, cells were incubated with 5 μg/mL (saturating concentration) 125I-mAb (Iodobeads; Pierce) in growth medium for 30 min at 4°C and washed. Cells were 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% Brij58 on ice. Sueces were centrifuged in a rotor at 100,000g for 45 min. Supernatants were collected and solubilized by incubation with 0.5% Triton X-100 for 1 h on ice. After centrifugation, supernatants were layered over a volume of 8.2, 140 mM NaCl, 2 mM EDTA, and this was layered over a volume of 60% sucrose. 25 and 5% sucrose layers were added to form a step gradient, and these gradients were centrifuged at 170,000×g for 18 h at 4°C. Fractions of 0.5 ml were collected from the top of the gradient, as well as the pellet, for Western blotting or assessment of radioactivity in a y counter.
Immunoprecipitations and Immunoblot—Jurkat cells (0.5–1.5 × 10^7 cells/point) were incubated on Ab-coated surfaces at 37 °C for 5–15 min, as indicated in the text. For analysis of PLCγ phosphorylation, cells were lysed in 1% Nonidet P-40, 0.5% deoxycholate, 50 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 10 μg/ml leupeptin and aprotonin, 10 mM β-glycerophosphate, 50 mM calyculin, and 250 μM sodium vanadate. Insoluble material was removed by centrifugation at 13,000 × g for 5 min. PLCγ was immunoprecipitated overnight by incubation at 4 °C with rabbit anti-PLCγ1 Ab (Upstate Biotechnology, Inc.) and GammaBind Plus-Sepharose (Amer sham Pharmacia Biotech). Immunoprecipitates were Western blotted with 4G10 antiphosphotyrosine mAb (Upstate Biotechnology, Inc.) as previously described (6). For the JNK assays, samples were processed according to the kit manufacturer's instructions (New England Biolab, Beverly, MA). Briefly, lysate from 2 × 10^6 cells/sample was mixed with glutathione S-transferase-c-Jun-bound glutathione-Sepharose beads, and a kinase reaction was performed with the bound material. Products were resolved by SDS-polyacrylamide gel electrophoresis on a 12.5% gel, transferred to polyvinylidene difluoride, and probed with a phospho-Ser63-specific rabbit anti-c-Jun polyclonal antibody. Equal loading of samples was confirmed by Coomassie staining prior to probing. The positive control for PLCγ phosphorylation was 0.5 mM pervanadate and for JNK activation was 20 ng/ml PMA/2 μM ionomycin for the last 15 min of treatment.

Production of IL-2—Production of IL-2 by adherent cells was assessed as described previously (6). Briefly, cells (1 × 10^6) were plated in wells coated with anti-CD47, anti-CD8, anti-FLAG, SIRPα1-Fc, or control mAbs together with various concentrations of anti-CD3 in RPMI medium. Anti-CD3 dilutions are indicated as percentages of hybridoma supernatant. After 18–24 h, IL-2 in harvested supernatants was measured by enzyme-linked immunosorbent assay using mAbs 5344.111 and B33–2 and recombinant human IL-2 (Phar mingen, San Diego, CA) to construct a standard curve or by incorporation of [3H] thymidine (0.4 μCi/well; specific activity, 6.7 Ci/mmol; ICN) by CTLL-2 cells as previously described (6).

Single Cell Fluorescence Calcium Measurements—Jurkat or JInB8 cells at 2 × 10^5 cells/ml in RPMI complete medium were incubated with 3 μM fura-2/acetoxymethyl ester at 37 °C for 20 min. The cell suspension was diluted 10-fold with complete medium and kept at 37 °C for another 20 min. After the incubation periods, cells were washed three times in ice-cold calcium buffer (25 mM HEPES, 125 mM NaCl, 5 mM KCl, 1 mM NaHPO_4, 0.5 mM MgCl_2, 1 mM CaCl_2, pH 7.4) and resuspended at 2.5 × 10^5 cells/ml in the same buffer and kept on ice until use. For evaluation of ligation of CD47 on a substrate with or without coligation of CD3, fura-2-loaded cells were washed and resuspended in calcium buffer and allowed to adhere to Ab-coated coverslips mounted in a Leiden coverslip dish in a PDMI-2 microincubator (Medical Systems Corp., Green ville, NY) at 37 °C while viewed through a Zeiss Axiosvert microscope. Samples were illuminated with light of alternating excitation wavelengths of 340 and 380 nm using a FL-4000 imaging system (Georgia Instruments, Roswell, GA), and the emission images were collected with a Dage MTD CCD72 camera and intensified connector connected to a Matrox MVP image processing card in a personal computer and stored on a Panasonic TQ3031F optical memory disc recorder. Recordings were made from the point of addition of cells to the coverslip with 340- and 380-nm images recorded every 10 s. The 10-min time point was used for routine analysis based on evaluation of the kinetics of the CD47 response (see Fig. 6).

Statistical Analysis—All experiments were repeated at least three times. Error bars in graphs depict the 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 f tests as appropriate. A p value of <0.05 was considered significant.

RESULTS

Ligation of CD47 Stimulates an Increase in F-actin—Previous studies showed that both Ig and MMS domains were required for CD47-stimulated cell spreading and for synergy with TCR in IL-2 synthesis (6, 12). This suggested that similar signaling pathways might activate both functions. Because the cytoskeleton is a critical determinant of cell shape and spreading as well as T cell activation, we hypothesized that CD47 ligation might signal actin cytoskeleton rearrangement. As shown in Fig. 1A, ligation of CD47 caused total cell F-actin content to increase more than when CD18 (β2 integrin LFA-1) was engaged. Because Jurkat cells do not spread on anti-CD18-coated surfaces (12), the F-actin content of these cells was used as a nonactivated control. CD47-induced increase in F-actin was less than that induced by optimal stimulation of CD3 (Fig. 1A). However, CD47 ligation increased F-actin content in both TCR-expressing and TCR-deficient Jurkat cells to the same extent (Fig. 1A) showing that, in contrast to CD47-induced Ca^2+ flux (8), this response is not TCR-dependent. Ligation of

![Image](347x316 to 516x730)

**Fig. 1.** Increase in F-actin by ligation of CD47. A, Jurkat cells expressing wt CD47, FLAG-MMS, or CD8-MMS were applied to surfaces coated with anti-CD18, anti-CD47, anti-FLAG, anti-CD8, or anti-CD28 antibodies for 1 h at 37 °C and fixed, and F-actin content was assessed as described under "Experimental Procedures." TCR-deficient Jurkat cells were allowed to adhere to anti-CD18- or anti-CD47-coated surfaces and assessed similarly. In all cases, F-actin content was normalized to the number of adherent cells by measuring DNA content; the F-actin in cells adherent to anti-CD18 was set at 100. Results indicate the means ± S.E. for five experiments. The diagrams above FLAG-MMS and CD8-MMS depict the form of the molecule ligated. Only CD47 and CD3 ligation resulted in increased F-actin (p < 0.05). B, CD47-deficient Jurkats transfected with wt CD47, CD47-CD7, or CD47-GPI were similarly treated and F-actin content in cells adherent via CD47 and CD18 compared. Actin content in CD18-adherent cells was 100. Results indicate the means ± S.E. for five experiments. Above each bar is a diagram of the mechanism for the association of each molecule with the plasma membrane. Only ligation of wt CD47 led to increased F-actin (p < 0.01). C, Jurkat cells expressing wt CD47 and FLAG-MMS were applied to surfaces coated with anti-CD47 or anti-FLAG along with varying amounts of anti-CD3. F-actin content was determined as previously described and is shown as fluorescence ratios without standardization to CD18 levels. Results are from a experiment (performed in triplicate) representative of four experiments with similar results.
the costimulatory molecule CD28, which does not stimulate spreading on its own, failed to stimulate a significant elevation of F-actin above the level of CD18-adherent cells. Ligation of CD47 mutants in which the Ig domain had been replaced either with the CD8 extracellular Ig domain (CD8-MMS) or the FLAG epitope (FLAG-MMS) using anti-CD8 or anti-FLAG failed to increase F-actin content (Fig. 1A), demonstrating that the Ig domain was necessary for this effect of CD47 ligation.

To determine whether a membrane-associated CD47 Ig domain was sufficient for actin polymerization, the CD47-deficient Jurkat line JinB8 (12) was studied (Fig. 1B). Whereas ligation of wild type CD47 transfected into JinB8-induced actin polymerization, ligation of mutants in which the MMS domain was replaced by the CD7 transmembrane domain (CD47-CD7) or by a signal for addition of a glycanphosphoinositol anchor (CD47-GPI) did not (Fig. 1B). Thus, both the Ig and MMS domains of CD47 are required for induction of net actin polymerization.

To determine whether F-actin increased when CD47 and CD3 were ligated simultaneously, F-actin was assessed when either wt CD47 or FLAG-MMS was coligated with CD3. Ligation of CD3 caused a concentration-dependent increase in F-actin content (Fig. 1C). In the absence of CD3 ligation, there was increased actin when CD47 was ligated compared with FLAG-MMS, as expected (Fig. 1A). The effects of CD47 and CD3 on F-actin were additive at low anti-CD3 concentrations, suggesting that this could contribute to CD47 synergy in T cell activation.

**Ligation of CD47 Stimulates PKCα Association with the Cytoskeleton**—Because PKCα association with the actin cytoskeleton may be important for its function (36), we evaluated the ability of CD47 to stimulate PKCα cytoskeletal association. Ligation of CD47 stimulated PKCα cytoskeletal association by 15 min after adhesion in the absence of CD3 ligation (Fig. 2A). In contrast, FLAG-MMS, CD8-MMS, and CD28 failed to stimulate PKCα translocation (Fig. 2A and data not shown). However, CD47-induced translocation was transient, because cytoskeletal-associated PKCα returned to base line by 30–40 min after stimulation (Fig. 2B and data not shown). These kinetics contrast with CD47-induced actin polymerization, which remained elevated for at least 1 h (Fig. 1 and data not shown). However, coligation of CD47 and subthreshold CD3 led to sustained PKCα translocation (Fig. 2B). This was not an effect simply of CD3 signaling, because its ligation together with CD28 or FLAG-MMS showed no PKCα association with cytoskeleton above the basal (BSA) level. The CD47 Ig domain was required for both its transient and sustained effects on PKCα, as shown by the failure of FLAG-MMS to induce PKCα translocation on its own (Fig. 2A) or in synergy with CD3 (Fig. 2B).

**SIRPa1 Ligation of CD47 Synergizes with CD3 for IL-2 Synthesis and Stimulates PKCα Cytoskeletal Association**—SIRPa1 is a recently described cell surface ligand for CD47 that is highly expressed on macrophages and dendritic cells but is not present on T cells (37, 38). CD47 interaction with SIRPa1 therefore has the potential to modulate antigen presentation to T cells. To determine whether SIRPa1 could induce CD47 signaling, Jurkat cells were allowed to adhere to surfaces coated with a SIRPa1-Fc fusion protein. Compared with the normal human IgG control, SIRPa1-Fc synergized with anti-CD3 for IL-2 synthesis, shifting the EC₅₀ for CD3 ligation about 25-fold (Fig. 3A). SIRPa1 ligation of CD47 also stimulated an increase in PKCα association with cytoskeleton in the absence of CD3 ligation (Fig. 3B). Thus, SIRPa1 ligation of CD47 induces PKCα-independent PKCα translocation and synergizes with TCR ligation for T cell activation, suggesting the possibility of a physiologic role for CD47/SIRPa1 binding during T cell/APC interactions.

**Association of CD47 with Membrane Rafts Correlates with Its Ability to Stimulate Cell Spreading and to Increase F-actin Content**—These data demonstrated that ligation of CD47 induces both actin polymerization and PKCα translocation to the cytoskeleton. These signals from CD47 required the Ig domain as well as the MMS domain. Because the ligation of CD8 or FLAG could not induce similar effects, the data demonstrate a function for the Ig domain independent of ligand binding. Thus, the Ig domain has a fundamental role in CD47 signaling in addition to its role in ligand recognition. Because the Ig domain is required for CD47 localization to membrane rafts in ovarian carcinoma cells (28), we hypothesized that a similar role for the Ig domain in T cells might account for its necessary contribution to CD47 signaling. To test this hypothesis, we first determined whether CD47 is concentrated in membrane rafts in Jurkat T cells. CD47 localized to rafts in both normal Jurkat cells and JinB8 cells transfected with wild type CD47 (Fig. 4). We also examined the structural requirements for CD47 association with membrane rafts in Jurkat cells. Although >65% of wild type plasma membrane CD47 associated with membrane raft fractions, CD47-CD7 failed to localize significantly to rafts, and the FLAG-MMS and CD8-MMS mutants also were markedly defective in raft localization (Fig. 4). Thus, both the MMS
and Ig domains of CD47 were necessary for its enrichment in plasma membrane rafts in Jurkat cells, identical to the requirements for spreading, actin polymerization, and TCR synergy. Furthermore, methyl-β-cyclodextrin, which disrupts raft organization (39), blocked CD47-induced cell spreading and the increase in F-actin in both TCR+ and TCR− Jurkat cells, and these effects were reversed by reconstitution of membrane cholesterol (data not shown). These data suggested that only CD47 in rafts could generate the signal for actin polymerization. To test whether raft localization was sufficient for CD47-induced actin polymerization, CD47-GPI was studied. This mutant localized even better to rafts than wild type CD47 (Fig. 4). However, the failure of CD47-GPI to increase F-actin (Fig. 1B) demonstrates that raft localization of the CD47 Ig domain is not a sufficient signal for actin polymerization. Altogether these data are consistent with the hypothesis that CD47-induced actin polymerization and PKC δ translocation are necessary for its effects on T cell activation and that CD47 localization to rafts is necessary but insufficient for these consequences of CD47 ligation.

**FLAG-MMS and CD8-MMS Can Synergize with CD3 for PLCγ Phosphorylation but Not JNK Activation—**CD47 has been shown to synergize with TCR for early steps in T cell activation and to stimulate a TCR-dependent Ca\(^{2+}\) flux (7, 8). To determine whether this synergy also required membrane raft localization of CD47, we assayed PLCγ activation by wt CD47 and the Ig and MMS mutants that failed to associate with membrane rafts, induce actin changes, or costimulate T cell activation (6, 12). Surprisingly, FLAG-MMS and CD8-MMS synergized to activate PLCγ1 as well as wt CD47 (Fig. 5A and data not shown). In contrast, neither FLAG-MMS or CD8-MMS could synergize with CD3 to activate JNK (Fig. 5A and data not shown), a required step in synergistic signaling for IL-2 synthesis. Wild type CD47 and CD28 costimulate JNK activation similarly (12). Neither the CD47-CD7 nor the CD47-GPI mutants could synergize with CD3 either for PLCγ phosphorylation (Fig. 5B) or JNK activation (data not shown).

**FLAG-MMS Costimulates \([\text{Ca}^{2+}]_i\), Flux—**To determine whether PLCγ phosphorylation by the FLAG-MMS mutant was capable of continuing the signaling cascade, single cell \([\text{Ca}^{2+}]_i\), recordings were made on cells plated onto surfaces coated with stimulatory anti-CD3 together with anti-CD47, anti-FLAG, or control mAbs. In cells expressing wt CD47, this led to a slow but sustained rise in \([\text{Ca}^{2+}]_i\), that lasted >40 min (Fig. 6 and data not shown). There was no increase in \([\text{Ca}^{2+}]_i\) when anti-CD18 (Fig. 6, 6A and B) or anti-CD35 (isotype matched nonbinding control; Fig. 6B) were substituted for anti-CD47. Ligation of CD47 alone did not induce a \([\text{Ca}^{2+}]_i\) flux, demonstrating that synergy with TCR signaling was required (Fig. 6B). Consistent with its synergy in PLCγ phosphorylation, ligation of FLAG-MMS costimulated \([\text{Ca}^{2+}]_i\) flux (Fig. 6C) that was sustained for >40 min. FLAG-MMS-dependent \([\text{Ca}^{2+}]_i\) flux occurred in JnB8 as well as Jurkat cells (data not shown), ruling out interaction between FLAG-MMS and endogenous CD47 as a cause of the \([\text{Ca}^{2+}]_i\) signaling. Thus, failure of FLAG-MMS to synergize for JNK activation and IL-2 synthesis is not due to the inability of activated PLCγ to continue the signaling cascade. The ability of FLAG-MMS to costimulate \([\text{Ca}^{2+}]_i\) demonstrates that neither efficient raft localization nor actin polymerization is required for CD47 synergy in PLCγ activation. Consistent with its inability to induce PLCγ phosphorylation, CD47-CD7 did not synergize with anti-CD3 to stimulate a \([\text{Ca}^{2+}]_i\) rise (Fig. 6D).
can CD47 engage a signaling cascade leading to actin polymerization and PKC\(\theta\) translocation. Because CD47 synergy for IL-2 synthesis has an identical structure-function profile as association with membrane rafts, actin polymerization, and PKC\(\theta\) translocation, it is tempting to speculate that these events are related. Certainly, PKC\(\theta\) plays an important role in activation of IL-2 synthesis in T cells (36, 41); both membrane rafts and actin polymerization have been implicated in this process as well (16, 42, 43). CD47 is known to cooperate with integrins in membrane rafts to activate heterotrimeric G proteins (28). However, we do not believe this is the mechanism by which the relevant signaling cascades are activated in T cells because (a) CD47 cooperation with TCR is independent of all integrins with which it is known to interact (6, 7) and (b) pertussis toxin does not inhibit synergy with TCR nor CD47-induced cell spreading.\(^2\) Whereas some heterotrimeric G proteins are pertussis toxin-independent, G protein signaling in Jurkat cells is potently inhibited by this toxin (44). Hence, we suggest that the most proximate steps in CD47 signaling in T cell membrane rafts do not involve heterotrimeric G proteins and remain to be discovered.

The TCR-independent signals that we have demonstrated do not occur outside rafts. However, the CD47 MMS domain can cooperate with TCR to increase \([Ca^{2+}]_i\), while apparently outside membrane rafts. Although the minimal amount of FLAG-MMS detected in rafts might be enough to effectively cooperate with CD3 to activate PLC\(\gamma\), it is clearly insufficient to activate actin polymerization or PKC\(\theta\) translocation or to synergize in JNK activation or IL-2 synthesis. Thus, we believe it most likely that the MMS domain outside rafts can engage signaling cascades important in PLC\(\gamma\) activation, and only the MMS domain inside rafts can induce the additional actin and PKC\(\theta\) signals required in T cell activation.

These discoveries have two implications. First, CD47-TCR cooperation has at least two discrete effects on T cell signaling pathways. Although synergy for Ca\(^{2+}\) elevation does not require CD47 cytoskeletal effects, JNK activation and IL-2 synthesis do. We suggest that this actin- and raft-independent PLC\(\gamma\) activation results from the previously reported synergy between CD47 and TCR for \(\zeta\) chain phosphorylation and zap-70 activation (6). The second implication of this work is that CD47 synergy in the early events of T cell activation are not sufficient to account for its effects on IL-2 synthesis. Thus, it is unlikely that the role of CD47 is simply to increase the efficiency of antigen presentation. Instead, our data support the hypothesis that CD47 cooperation with TCR for IL-2 synthesis results from its independent cytoskeletal signaling within raft domains. It is likely that the CD47-related F-actin and PKC\(\theta\) are

\(^2\) R. A. Rebres, M. I. Reinhold, and E. J. Brown, unpublished data.
were incubated with anti-CD3 (0.1%) coimmobilized with 1 domain of CD47, but PLC\textsubscript{g} experiments. Only intact CD47 shows synergy with CD3 in PLC\textsubscript{g} or anti-CD18 control mAbs on plates for 10 min at 37 °C. PLC\textsubscript{g} independent signaling. The requirement for the extracellular

IL-2 secretion proceeds by an entirely distinct mechanism. This could enhance TCR activation of PKC, and

phosphorylation (\textsuperscript{c}SIRP\textsubscript{CD47}). This could enhance TCR activation of PKC, and downstream events, like JNK activation, could proceed more efficiently. In Jurkat cells, at least, CD28 does not increase cytoskeletal association of PKC\textsubscript{B}, implying that its synergy of IL-2 secretion proceeds by an entirely distinct mechanism.

Structure-function studies demonstrated a requirement for both the MMS and the Ig domains of CD47 for effective TCR-independent signaling. The requirement for the extracellular

Ig domain in signal transduction is quite unusual, because signaling is thought to be a property of the cytoplasmic or intramembrane sequences of most receptors. The explanation for this unusual requirement appears to be that the Ig domain is required for appropriate localization of CD47 to membrane rafts. Why this should be so is unclear, because most signals for raft localization in transmembrane molecules lie within the transmembrane domain or the juxta-membrane cytoplasmic tail. The requirement for the Ig domain suggests that either it interacts with another membrane molecule to stabilize CD47 raft association or the presence of the Ig domain influences the conformation of the MMS domain for its association with rafts. If lateral association of intact CD47 with another membrane protein mediates raft association, this protein must be ubiquitous, because CD47 localizes to rafts in ovarian carcinoma (OV10), melanoma (C32), and neutrophils as well as Jurkat cells. Although we cannot rule this out, we favor the hypothesis that the CD47 Ig domain directly influences the ability of the MMS domain to associate with rafts. The reduced localization of CD47 to rafts in JinB8 versus Jurkat cells may represent a reduced general stability of raft domains from JinB8 to the detergent conditions used, because even CD47-GPI shows some extra-raft distribution in these cells. Reduced raft organization in JinB8 cells may also contribute to the reduced CD3 responsiveness of this line. The MMS domain certainly is required for CD47 signaling, because its deletion abolishes both raft-dependent and raft-independent functions of CD47. The primacy of this domain in CD47 signaling is logical, because the C-terminal cytoplasmic tails of CD47 are quite short and have no known signaling motifs (1). How the MMS domain signals, either in or out of rafts, is entirely unknown. Initial mutagenesis experiments have not revealed sequences within the predicted transmembrane domains or intracytoplasmic loops that have affected CD47 function in Jurkat cells. It is interesting that in an assay of T cell adhesion to endothelium, CD47 lacking most of its MMS domain still functioned normally.

A potentially significant role for CD47 in T cell response to antigen presentation \textit{in vivo} is suggested by the finding that SIRP\textsubscript{CD47}, a CD47 ligand highly expressed on dendritic cells (38), can activate CD47 signaling. It is likely that SIRP ligation of CD47 can increase the sensitivity of T cells to limiting doses of antigen. Although the B7 ligands for CD28 are not constitutively expressed on APC but require some cell activation to achieve a significant level of expression, SIRP expression appears constitutive. Thus, SIRP-CD47 interaction is likely to have its greatest effect on T cell activation during initial antigen presentation prior to generation of an inflammatory response that would activate B7 expression. Perhaps this is the reason that CD47-deficient mice have approximately half the number of peripheral T cells as their wild type littermates.

Several adhesion molecules may synergize with TCR in a manner similar to that of CD47. For example, in some T cells, LFA-1 also synergizes with TCR in activation, and this requires cell adhesion to an APC or experimental surface presenting both TCR ligand and ICAM-1 (9). Reorganization of the cortical actin cytoskeleton is stimulated by engagement of LFA-1, and this plays an important role in the spatial organization of Ag receptors during T cell activation (18, 47). We postulate that adhesion molecules in general may contribute to T cell activation through similar rearrangement of the cytoskeleton and signaling factor localization. Because many signaling molecules interact directly or indirectly with cytoskel-
et al., the role of adhesion molecules can be seen as concentrating the elements of specific signaling cascades at the sites of TCR ligation.

In conclusion, we have shown that CD47 in Jurkat cells can generate TCR-independent signals upon ligation with antibody or its known intercellular ligand, SIRPα1. Most prominently, CD47 induces the translocation of PKCζ to the cytoskeleton, an effect that in turn requires an increase in actin polymerization. This signaling, but not synergy with TCR for Ca²⁺ flux, requires CD47 localization to membrane rafts, presumably for this complex molecule to engage the appropriate signaling cascades. CD47 and TCR cooperate to sustain the cytoskeletal association of PKCζ, perhaps because TCR signaling generates the lipid mediators necessary to activate the enzyme, leading to more stable association with the plasma membrane and the associated cortical actin cytoskeleton. This synergy likely is responsible for the cooperation between CD47 and TCR in JNK activation and IL-2 transcription. Although much has been written about how signal transduction cascades affect the cytoskeleton, this may be an example of how modulation of the cytoskeleton can affect signal transduction. This could be a general mechanism by which effects on actin polymerization can modulate signaling cues from many receptors and may be the mechanism by which many adhesion receptors modulate inputs from the TCR.

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