

Peptide-Mediated Inhibition of Neutrophil Transmigration by Blocking CD47 Interactions with Signal Regulatory Protein α^1

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CD47, a cell surface transmembrane Ig superfamily member, is an extracellular ligand for signal regulatory protein (SIRP α). Interactions between CD47 and SIRP α regulate many important immune cell functions including neutrophil (PMN) transmigration. Here we report identification of a novel function-blocking peptide, CERVIGTGWVRC, that structurally mimics an epitope on CD47 and binds to SIRP α . The CERVIGTGWVRC sequence was identified by panning phage display libraries on the inhibitory CD47 mAb, C5D5. In vitro PMN migration assays demonstrated that peptide CERVIGTGWVRC specifically inhibited PMN migration across intestinal epithelial monolayers and matrix in a dose-dependent fashion. Further studies using recombinant proteins indicated that the peptide specifically blocks CD47 and SIRP α binding in a dose-dependent fashion. Protein binding assays using SIRP α domain-specific recombinant proteins demonstrated that this peptide directly bound to the distal-most Ig loop of SIRP α , the same loop where CD47 binds. In summary, these findings support the relevance of CD47-SIRP α interactions in regulation of PMN transmigration and provide structural data predicting the key residues involved on the surface of CD47. Such peptide reagents may be useful for studies on experimental models of inflammation and provide a template for the design of anti-inflammatory agents. *The Journal of Immunology*, 2004, 172: 2578–2585.

CD47 is an Ig superfamily transmembrane glycoprotein that is widely expressed in most cells and tissues. The complete cDNA sequence encodes a protein of 305 aa (isoform 2) (1, 2) that contains an N-terminal extracellular domain that structurally belongs to the Ig variable-like (IgV)⁴ superfamily, a transmembrane domain comprising of three or five transmembrane segments and a hydrophilic intracytoplasmic tail. CD47 has been shown to play an important role in many different immune cell functions including neutrophil (PMN) transmigration in response to inflammatory stimuli (3). A potent CD47 mAb, C5D5, was identified and is capable of inhibiting PMN migration across collagen-coated filters, endothelial monolayer, and intestinal epithelium without inhibiting β_2 integrin-mediated adhesion (3). Subsequent studies with CD47 knockout mice confirmed the importance of CD47 in regulating PMN recruitment to sites of infection in vivo (4). These data and more recent findings (5) suggest that tissue-expressed CD47 may serve to “fine tune” the acute inflammatory response by regulating the rate of PMN migration toward sites of injury or infection. Despite these important observations,

the mechanism by which CD47 regulates PMN migration is not clear. In other cell systems, CD47 has been shown to functionally and physically associate with β_3 and β_1 integrins, and the C-terminal cell binding domain of thrombospondin-1 (6–9). However, these interactions do not appear to modulate PMN migration (5). Consistent with studies on other Ig superfamily proteins indicating that IgV-like domains are important in cell surface adhesive functions (10, 11), CD47 has also been shown to be an extracellular receptor of signal regulatory protein (SIRP α) (12–14). In support of this, CD47 purified from multiple human tissues and recombinant CD47 extracellular domain binds to the extracellular domain of SIRP α 1 in vitro (15). Furthermore, we have shown that CD47-SIRP α interactions play an important regulatory role in PMN transmigration (15).

SIRP α and SIRP β are a family of transmembrane glycoproteins richly expressed in PMN, macrophages, and certain other hematopoietic cell types (13, 16, 17). Primary structural analyses predict that these Ig superfamily proteins contain an N-terminal extracellular domain comprising multiple Ig-like loops, a single transmembrane segment and a C-terminal intracellular domain (16). CD47 binds to the most distal Ig loop of SIRP α (17, 14) and SIRP α -CD47 interactions have been implicated in multiple cellular processes, including PMN and monocyte transmigration (15, 18), human melanoma cell and CHO cell migration (19), macrophage multinucleation (20), self-recognition of RBC and sickle cell clearance (21, 22), T cell responses, and dendritic cell maturation (23).

In this study, we used phage display techniques to identify functional domain(s) on CD47 by panning on the blocking CD47 mAb C5D5. We obtained a high affinity C5D5 binding sequence, CERVIGTGWVRC (termed C5D5.1). Evidence indicates that the structure of CERVIGTGWVRC represents a functional domain on CD47 that binds to SIRP α and inhibits PMN transmigration. A comparison of the IgV loop of CD47 with known Ig structures suggests that key residues on this peptide may lie in close proximity on CD47 surface as part of a structural domain involved in regulation of PMN transmigration by interactions with SIRP α .

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⁴ Abbreviations used in this paper: IgV, Ig variable-like; PMN, neutrophil; SIRP α , signal regulatory protein; AP, alkaline phosphatase.

Materials and Methods

Abs, phage libraries, and other reagents

Anti-CD47 mAbs C5D5 and isotype-matched control mAb J10.4 (anti-JAM) were described previously (3, 5). Fab of C5D5 were prepared by papain digestion of C5D5 IgG, followed by removal of Fc by protein A chromatography (Lampire Biological Laboratories, Pipersville, PA). Rabbit polyclonal anti-M13 phage Ab was provided by Dr. J. Burritt (Montana State University, Bozeman, MT). Three random phage-display libraries used in this study were generated in our laboratory including one linear random nona-peptide M13 phage library termed LL9 and two structurally constrained libraries displaying hexa- and deca-peptide loops, termed CL6 and CL10 respectively (24). The latter libraries were constructed by introducing DNA bases coding for two cysteine residues at both N- and C terminus of the random peptides (24). Synthetic peptides were produced by solid phase synthesis and purified by HPLC (purity >90%) at the Microchemical Facility, Emory University (Atlanta, GA). Peptides oxidation (>95%) was performed by dissolving in 0.1 M NH₄HCO₃, pH 8.0, followed by stirring for 48 h at room temperature. Cyclization was determined by MALDI and a negative Elman's test. Human CD47 was immuno-purified using C5D5 or PF3.1-Sepharose as described previously (3, 15).

Panning phage libraries on mAbs and plaque lift assays

Isolation of phage bearing epitopes for C5D5 was performed as follows: 10¹⁰ M13 phage from LL9, CL6, or CL10 library were mixed with C5D5-Sepharose (2–3 mg/ml) in 1 ml phage buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5% Tween 20 (v/v), 1 mg/ml BSA) and rotated overnight (4°C). After washing, bound phage were eluted with 1 ml 0.1 M glycine, pH 2.2, and immediately neutralized with 150 mM NaCl, 10 mM HEPES, pH 7.5. Titers of eluted phage were determined accordingly (25), followed by amplification in *Escherichia coli* strain K91 cells on LB-agar containing 75 µg/ml kanamycin as described previously (26). The bacterial colonies were then scraped, lysed, and phage were purified. After titration, ~10¹⁰

phage obtained from the first round amplification were mixed with C5D5-Sepharose to perform a second round of panning. A total of three rounds of panning were performed with each library. For controls, three rounds of panning were performed using Sepharose conjugated with normal mouse IgG. Eluted phage from third round panning were plated on LB-agar containing K91 cells at a density of ~100 plaques per plate, lifted onto nitrocellulose membrane and probed with mAb C5D5 (2 µg/ml) for 1 h. The bound mAb was detected by HRP-conjugated rabbit anti-mouse IgG followed by ECL detection. Positive C5D5-binding phage plaques were picked, amplified, and phage DNA was prepared and sequenced using the primer described previously (24).

Recombinant proteins

Recombinant CD47 and SIRPα1 extracellular domain fusion proteins CD47-alkaline phosphatase (AP) and SIRPα1-GST were produced as described previously (13, 15–17). Ig loop specific SIRPα1 and Fc fusion proteins were generated. The DNA sequences that contain Ig loop 1 (Ig loops are numbered from the membrane distal as loop 1), Ig loops 1+2, and Ig loops 1+2+3 were PCR amplified using sense primer SP1 (atgataagctccgcccagccatgagcccgc) and antisense primers SP4 (ttgat tagatctggtgaagctcactgtgtgctg), SP3 (ttgattagatctggtgacattcactgttctc), and SP2 (ttgattagatctagttctcagcggcgatt), respectively. After digestion with *Hind*III and *Bgl*III, these DNA fragments were cloned into predigested pCDNA3.1.Fc vector (digested with *Hind*III and *Bam*H I) that contained a rabbit IgG Fc fragment (27). To construct fusion proteins containing Ig loop 2, 2+3, and 3, PCR was performed to first amplify DNA sequence coding for SIRPα1 signal peptide by primers SP1 and SP7 (5'-aggatccagcagccaggcaggcagcggcgag), digested with *Hind*III and *Bam*HI, and cloned into the same pCDNA3.1.Fc vector. A positive clone was selected and the plasmid named pCDNA.Fc-S. PCRs were then performed to amplify the Ig2, Ig2+3, and Ig3 loops by primer pairs SP8 (5'-aggatccagcaca cagtgaacttacc)/SP3, SP8/SP2, and SP9 (5'-aggatccagagaaccaggtgaatgt

Table I. Peptide sequences obtained from panning mAb C5D5 with cysteine constrained (CL10 and CL6) phage display libraries^a

Panning Selected Phages	Binding to C5D5 (by ELISA)	Peptide Synthesized	
		Name	Solubility
<i>From CL10 library:</i>			
CERVIGT <u>GWVRC</u> ×6	++	C5D5.1	>10 mM
CHRVPGH <u>GWVRC</u> ×5	++	C5D5.12	>10 mM
CSWQHQD <u>GWVWC</u>	++		
CVPVCRE <u>GWCGC</u> ×3	n.t.		
CYKSMD <u>GWVPC</u> ×3	++		
CVENV <u>DGWTVPC</u> ×2	++		
CRVPET <u>GWVVC</u>	n.t.		
CRLMLN <u>GWVPC</u>	n.t.		
CCRD <u>GWCHHDWC</u>	++		
CCRE <u>GWCGDGLC</u>	n.t.		
CGWRN <u>SFGQSLC</u> ×32	++	C5D5.2	>10 mM
CGWRN <u>ALQVVC</u>	n.t.		
CGWRN <u>LEGGSVVC</u>	n.t.		
CGWRD <u>DSGQSMC</u>	n.t.		
CRRVIGR <u>VGCGC</u>	++	C5D5.3	>10 mM
<i>From CL6 library:</i>			
WCRGG <u>WVC</u> ×4	++		
WCKSG <u>WVC</u> ×2	++		
CLCAE <u>GWVC</u> ×4	++		
CHPGT <u>GWVC</u> ×3	++		
SYCRE <u>GWVC</u>	+		
CGCRD <u>GWVC</u>	+		
CLCHG <u>GWVC</u>	+		
WCVK <u>GWVC</u> ×2	n.t.		
CRD <u>GWVCYS</u>	NS		
CVAILKDC	–		
<i>Controls peptides for C5D5.1:</i>			
ERVIGTGWVR		C5D5.4	>10 mM
CGVRTWRGVIEC		C5D5.5	2 mM
CHRVIGTGWVRC		C5D5.11	>10 mM

^a ELISA results quantifying binding of such phage to mAb C5D5 are also shown with OD values ++ (>1.0), + (<1.0), – (background), and n.t. (not tested). Consensus residues are either in bold or underlined. Sequences that were selected for peptide synthesis and solubility in aqueous phase are listed.

cacc)/SP2, respectively. After digestion with *Bam*HI and *Bgl*II, the fragments were cloned into the *Bam*HI digested pCDNA.Fc-S plasmid. Positive clones were checked for proper orientation by restriction digestion with *Hind*III and *Bam*HI. The correctly oriented sequences yielded DNA fragments with sizes of ~400 bp for Ig2 and Ig3, and ~700 bp for Ig2 + Ig3.

ELISAs

Ab-phage binding assays were performed by coating microtiter plate wells with Abs (2 μ g/ml) in HBSS. After blocking with 1% BSA, the wells were incubated with amplified phage clones (10^9) for 1 h. After washing, binding was detected with rabbit anti-M13 phage Ab (1:500 dilution) and peroxidase conjugated goat anti-rabbit secondary. To test synthetic peptide binding to mAbs, wells were coated with peptide (2 mM in 50 μ l HBSS) for 10 h to allow the peptide to effectively bind to the plate. After blocking with 1% BSA, mAb (2 μ g/ml) were incubated for 30 min followed by washing and detection with peroxidase-conjugated rabbit anti-mouse secondary. To test peptide binding to recombinant proteins, peptide-coated wells were incubated with 2 μ g/ml recombinant proteins (in HBSS) for 30 min. After washing, binding of GST fusion protein was detected by a goat anti-GST Ab (Amersham, Arlington Heights, IL) and peroxidase-conjugated rabbit anti-goat secondary. Binding of AP fusion protein was directly assayed for alkaline phosphatase activity. Binding of Fc fusion proteins were detected by peroxidase-conjugated goat anti-rabbit secondary Ab. For peptide competition assays, microtiter plates were coated with purified human CD47 (purified from PMN (3, 15), 1:50 dilution in HBSS). After blocking, C5D5 Fab was added (0.1–0.5 μ g/ml) in the absence or presence of increasing concentrations of peptide. After washing the wells, binding was detected with peroxidase-conjugated rabbit anti-mouse IgG (Fab)₂ Ab (The Jackson Laboratory, Bar Harbor, ME). Similar competition experiments were done to assay peptide inhibition of CD47-SIRP α binding. In these experiments, SIRP α -GST (2 μ g/ml) coated wells were incubated with CD47-AP (0.5 μ g/ml) in the absence or presence of increasing concentrations of peptide. Binding of CD47-AP was then detected by assay for alkaline phosphatase activity.

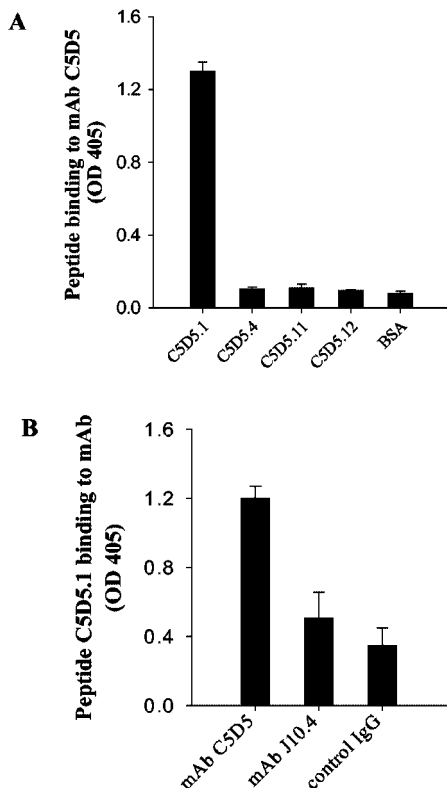


FIGURE 1. Peptide C5D5.1 binds directly and specifically to mAb C5D5. *A*, As detailed in *Materials and Methods*, binding of mAb C5D5 to microtiter wells coated with peptide C5D5.1 (oxidized) or control peptides C5D5.4, C5D5.11 (oxidized), and C5D5.12 (oxidized) is shown. *B*, Peptide C5D5.1 (oxidized) specifically binds to mAb C5D5 but not control mouse IgG. The data represent the mean and SD of triplicate wells per condition in one of three experiments.

PMN transmigration assay

Human PMN were isolated from peripheral blood of normal volunteers using a gelatin sedimentation method (3). PMN migration across collagen-coated transwell filters was performed as previously described (5). Briefly, permeable polycarbonate filters (5 μ m pore size) with a surface area of 0.33 cm² (Costar, Cambridge, MA) were coated with a solution of collagen purified from rat-tails (~50 μ g/ml in 70% ethanol) and allowed to dry. PMN (1×10^6) were preincubated with either peptide C5D5.1 or control peptide C5D5.4 before adding to the upper chamber in 150 μ l HBSS. Transmigration was initiated by the addition of fMLP to a final concentration of 100 nM into the lower chamber followed by incubation at 37°C. PMN migration into the fMLP-containing lower chambers was quantified by myeloperoxidase assay as previously described (3). Basolateral to apical PMN transepithelial migration assays were performed using inverted monolayers of T84 intestinal epithelial cells grown on collagen-coated transwell filters as previously described (3).

Results

Identification of mAb C5D5 binding sequence on CD47 by phage display

In this study, experiments were performed to identify the functional peptide domain on CD47 that mimics the epitope of the inhibitory anti-CD47 mAb C5D5. Phage display libraries were panned over the Sepharose conjugated with the mAb C5D5. After three rounds of Ab panning/amplification and plaque-lift assays (detailed in *Materials and Methods*), a total of 80 positive C5D5-binding phages were selected from cysteine constrained hexa- and deca-peptide libraries CL10 and CL6. However, no conclusive positive binding phage was obtained from the linear nona-peptide phage library LL9. Table I lists the deduced amino acid sequences from the positive C5D5-binding phages. As can be seen, 15 different amino acid sequences were recovered from 60 positive C5D5-binding CL10 phage with 5 sequences produced more than once (for example, phage bearing the sequence C5D5.1 were recovered six times). Ten different sequences were obtained from the CL6 of 20 positive phages (Table I).

Analysis of the C5D5 selected phage sequences in Table I revealed two major consensus sequences: C-(X)-R-(V/X-X-X)-D/E/N/T-G-W-V/C-R/X-(X)/(X-X-X)-C (conserved residues are in bold; residues with “/” are interchangeable; residues in () are either

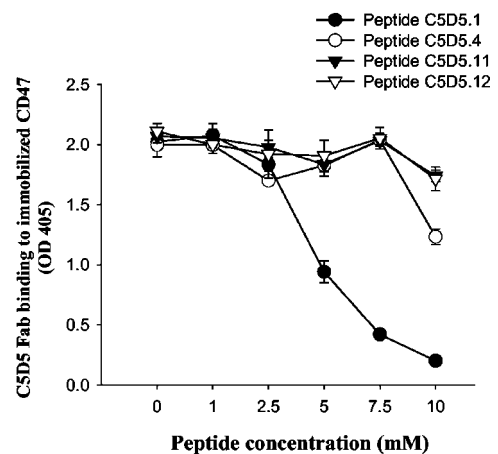


FIGURE 2. Inhibition of CD47-C5D5 Fab binding by peptide C5D5.1. Microtiter plate wells were coated with purified CD47 (from human PMN) for 2 h. After blocking, C5D5 Fab (1 μ g/ml) was added into wells without or with increasing amounts of peptide C5D5.1 (●), control peptides C5D5.4 (○), C5D5.11 (▲), or C5D5.12 (△). Peptides C5D5.1, C5D5.11, and C5D5.12 were oxidized. Binding of C5D5 Fab to CD47 was detected by a peroxidase-conjugated secondary Ab. The data represent the mean \pm SD of triplicate wells per condition in one of three experiments.

present or absent, X represents a variable residue) and C-G-W-R-N/D-X-X-G-Q-S-V/L-C from CL10 library. Only one CL10 phage sequence CRRVIGRVGCGC had no GW consensus (Table I). A consensus sequence C-R/X-E/D/T/G-G-W-C, which resembles the first consensus motif of CL10 phage sequences, was obtained from CL6 phage (Table I).

To further assess the binding of the consensus sequences from CL10 phage, three peptides with representative sequences were synthesized corresponding to those listed in Table I. Peptide CERVIGTGWVRC, termed C5D5.1, was synthesized because phage bearing this sequence produced the strongest positive signal continuously in the plaque-lift assay during phage isolation. Peptide CGWRNSFGQSLC (named C5D5.2) was chosen because its high frequency of recovery (Table I). We also synthesized peptide CRRVIGRVGC (C5D5.3) because it represents the group of sequences that lacks GW doublets. Given that the IgV structure contains a highly conserved tryptophan (W) (10, 11), we suspected that this residue might be important for CD47 function. All of these peptides were oxidized *in vitro* and analyzed by MALDI indicating that in each case, oxidation was achieved with intramolecular cyclization (disulfide bond formation) strongly favored over intermolecular oligomerization (results not shown).

These synthetic peptides were then tested for affinity in binding to mAb C5D5. As shown in Fig. 1A, peptide C5D5.1 bound most readily to mAb C5D5 and competitively blocked all phage binding to C5D5 at IC₅₀ values of 0.06–37 μ M depending on the individual phage tested (results not shown). Peptide C5D5.2 showed lower binding affinity but was able to block phage binding to C5D5 at concentrations above 1 mM (data not shown). Peptide C5D5.3, which has no GW residues, did not exhibit direct binding to C5D5 and only showed inhibition in phage binding at concentrations over 10 mM (not shown). To further access the structure and residues of peptide C5D5.1, several control peptides were also synthesized. As shown in Fig. 1A, no binding was observed with control peptide C5D5.4, which retains the core amino acid sequence

but is not cyclized because it lacks the two Cys residues of C5D5.1 (Table I). Replacing the negatively charged residue glutamic acid (E) to histidine (H) on C5D5.1 (peptide C5D5.11) also abrogated the binding ability (Fig. 1A). Fig. 1B shows results indicating that peptide C5D5.1 (CERVIGTGWVRC) binds specifically to mAb C5D5 but not other IgGs.

Experiments were also performed to test whether this peptide could compete with CD47 in binding to C5D5. As shown in Fig. 2, peptide C5D5.1 competitively inhibited C5D5 Fab binding to CD47 with an IC₅₀ of 3.8 mM. In contrast, control peptides, including C5D5.11 and C5D5.12 had no inhibition at concentrations up to 10 mM and C5D5.4 only displayed slight inhibition at a concentration of 10 mM (Fig. 2). These results suggest that peptide C5D5.1 could imitate the natural epitope of C5D5 on CD47. Although the inhibitory concentrations determined for C5D5.1 were relatively high (IC₅₀ 3.8 mM), they are comparable to those reported in other epitope mapping studies using hexa- and heptamer peptides (28, 29).

Peptide CERVIGTGWVRC inhibits PMN transepithelial migration

Experiments were performed to test peptide C5D5.1 for effects on fMLP-driven PMN transmigration across confluent T84 intestinal epithelial monolayers using a well characterized transwell assays (3, 5, 15). As shown in Fig. 3A, compared with the nonpeptide treatment control in which 43.4 \pm 6.1% of total applied PMN transmigrated after 1 h, in the presence of 1 mM peptide C5D5.1, PMN transmigration was decreased to 16.3 \pm 2.3% (~60–70% inhibition). In the meantime, the same concentrations of control peptides C5D5.4, C5D5.11, and C5D5.12 did not significantly affect PMN migration (39.2 \pm 3.1%, 35.4 \pm 7.8%, and 32.3 \pm 2.5% migrated in the presence of C5D5.4, C5D5.11, and C5D5.12, respectively). Fig. 3B shows the dose-dependence of C5D5.1-mediated inhibition of PMN transmigration across epithelial monolayers. As can be seen, peptide C5D5.1 inhibited PMN transmigration

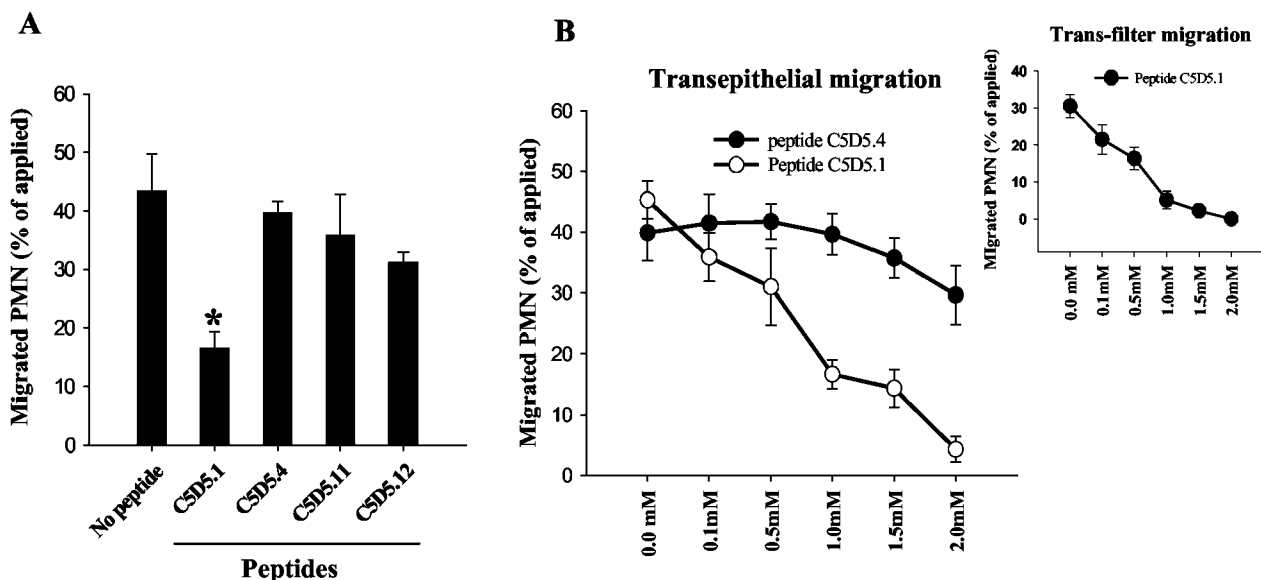


FIGURE 3. Effects of synthetic peptides on PMN transmigration. *A*, As detailed in *Materials and Methods*, PMN migration across T84 epithelial monolayers in the absence of 1 mM peptide C5D5.1 was compared with migration in the absence of peptides, or presence of control peptides C5D5.4, C5D5.11, or C5D5.12 (1 mM each). Migrated PMN after 1 h was assayed by measuring MPO (*, $p < 0.01$). *B*, Peptide C5D5.1 dose-dependently inhibits PMN transmigration across T84 epithelial monolayers. Peptide C5D5.1 (○) and control peptide C5D5.4 (●) were used at concentrations ranging from 0.1 to 2 mM and PMN were allowed to migrate for 1 h. Similar inhibitory results were observed using C5D5.1 in PMN transmigration across collagen-coated filters (*inset*). Peptides used including C5D5.1, C5D5.11, and C5D5.12 were oxidized. Data represents mean \pm SD of three transwells per condition in one of three experiments.

at concentrations of 0.5 mM and reached complete inhibition at concentrations of 2 mM or greater. Similar C5D5.1 inhibition profiles were observed for PMN transmigration across collagen-coated permeable filters (Fig 3B, *inset*). In the same assays, however, control peptide C5D5.4 demonstrated only minimal effect at a concentration of 2 mM (Fig. 3).

Peptide CERVIGTGWVRC structurally resembles SIRP α binding domain on CD47

Previously, we demonstrated that CD47-SIRP α interaction plays an important role in regulating PMN transmigration and this CD47-SIRP α interaction was blocked by mAb C5D5 (15). We explored the possibility that SIRP α binding site on CD47 overlaps with the epitope for mAb C5D5 by testing the effects of peptide C5D5.1 on CD47-SIRP α binding interactions. In vitro CD47-SIRP α binding experiments were conducted using the extracellular domain recombinant proteins of SIRP α 1 (SIRP α 1-GST) and CD47 (CD47-AP) (15). As shown in Fig. 4A, control peptides

C5D5.11, C5D5.12, and C5D5.4 (each used at 2 mM) demonstrated no or minimal (C5D5.4) inhibition in CD47-AP binding to SIRP α 1-GST compared with control binding in the absence of any peptide. In contrast, the same concentration of peptide C5D5.1 inhibited CD47-AP binding to SIRP α 1-GST by ~45%. In Fig. 4B, peptide C5D5.1 inhibited CD47-SIRP α binding in a dose-dependent manner such that 4 mM peptide C5D5.1 completely inhibited CD47-AP binding to SIRP α 1-GST, while peptide C5D5.4 only exhibited partial inhibition at the same concentration (Fig. 4B). Peptides C5D5.11 and C5D5.12 were not inhibitory at concentrations up to 4 mM (results not shown).

To confirm that peptide C5D5.1 inhibits SIRP α -CD47 interaction by competitively binding to SIRP α but not CD47, peptide C5D5.1 was immobilized on microtiter plates and assayed for binding to SIRP α 1-GST and CD47-AP. As shown in Fig. 4C, peptide C5D5.1 selectively bound to SIRP α 1-GST, but not CD47-AP (ruling out the possibility of C5D5.1 and CD47 homophilic interactions). Further binding studies using Ig domain-specific SIRP α 1

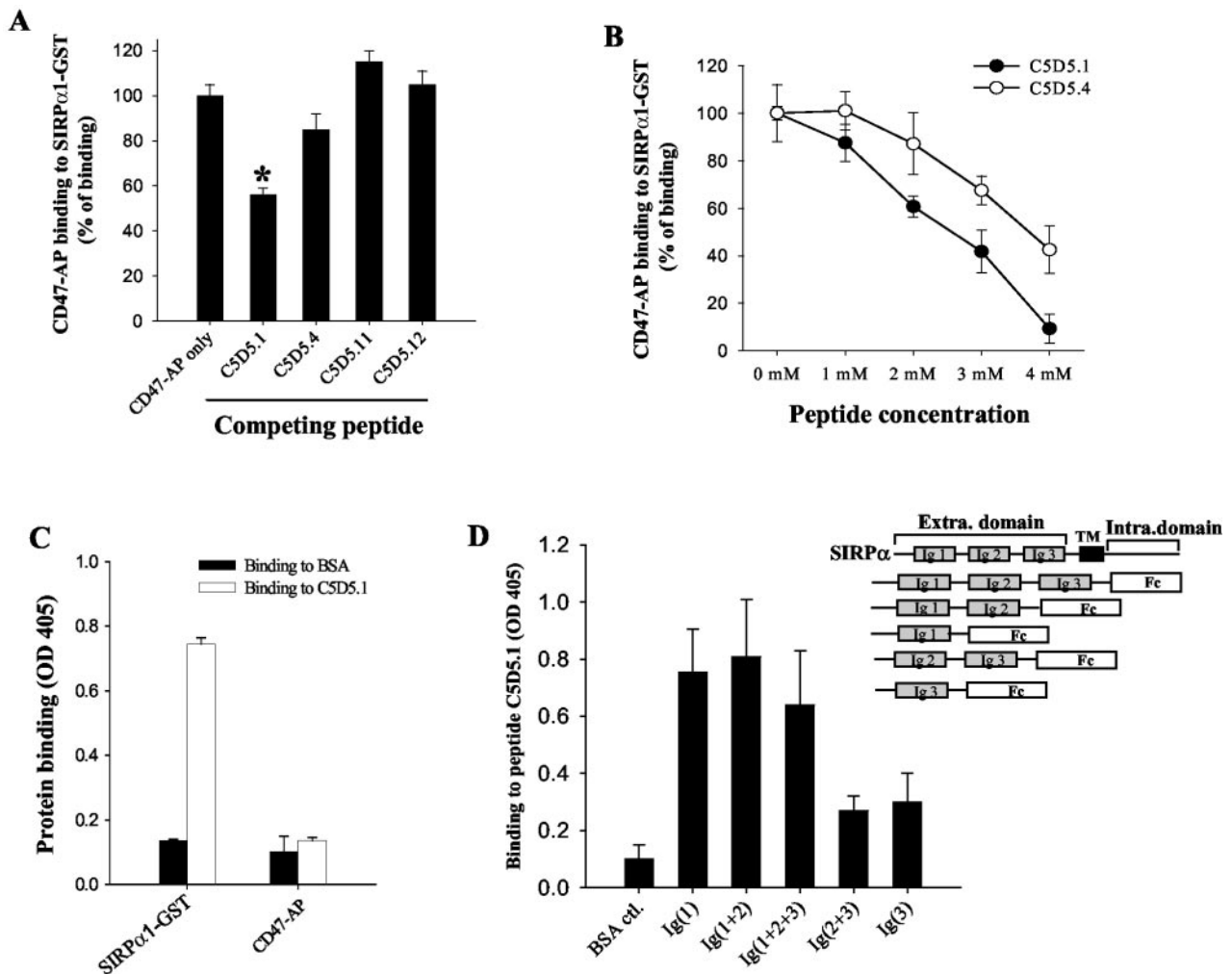


FIGURE 4. A, Inhibitory effects of peptide C5D5.1 on CD47-SIRP α binding. Microtiter plates were coated with SIRP α 1-GST fusion protein (2 μ g/ml). After blocking, CD47-AP was added in the absence or presence of synthetic peptide C5D5.1, control peptides C5D5.4, C5D5.11, or C5D5.12 (used as 2 mM each). After washing, binding of CD47-AP to SIRP α 1-GST was detected by assay of AP (*, $p < 0.01$). B, Dose-dependent inhibition of CD47-SIRP α 1 binding by adding increasing amounts of peptide C5D5.1 and control peptide C5D5.4 as in A. C, Peptide C5D5.1 binds directly to SIRP α 1-GST. Microtiter plates were coated with 2 mM peptide C5D5.1 or 1% BSA. After blocking, wells were incubated with either SIRP α 1-GST or CD47-AP (5 μ g/ml) and binding was detected by a goat anti-GST Ab (for SIRP α 1-GST) or by assay of AP (for CD47-AP). D, Peptide C5D5.1 binds to the membrane distal Ig loop (Ig1) of SIRP α 1. Recombinant Fc fusion proteins containing different Ig loop(s) of SIRP α 1 extracellular domain were generated as detailed in *Materials and Methods* and illustrated as in the figure inset (Fig 4D, *inset*). Binding of these Fc fusion proteins to immobilized peptide C5D5.1 was measured by a peroxidase conjugated anti-rabbit IgG. Peptides used including C5D5.1, C5D5.11, and C5D5.12 were oxidized. All data represent the mean \pm SD of triplicate wells per condition in one of three experiments.

fusion proteins showed that (Fig. 4D) peptide C5D5.1 specifically bound to the most distal Ig loop (loop 1) of SIRP α 1 and any fusion proteins containing Ig loop 1. This is inconsistent with our results using CD47-AP in binding to Ig domain specific SIRP α 1 fusion proteins (results not shown), and in agreement with previous reports that the CD47 binding site is located at the most distal Ig loop of SIRP α (17, 14). Taken together, these results suggest that the amino acid sequence in peptide C5D5.1 mimics, at least in part, the surface epitope on CD47 that binds to SIRP α .

Discussion

CD47 binds SIRP α to regulate multiple cellular functions via interactions between extracellular Ig-like domains (12, 13, 15, 17, 30). Although recent studies have shown that the membrane-distal Ig loop of SIRP α extracellular domain is important in binding to CD47 (17, 14), the specific peptide domains involved in binding on either CD47 or SIRP α are unknown. Because the extracellular domain of CD47 contains only one IgV-like loop, we initially tried to define the essential residues on the CD47 extracellular domain by site-directed mutagenesis. These studies, however, were inconclusive. We observed that mutation of the highly conserved “invariant” tryptophan (W⁵⁸) (10) to alanine within the IgV-like domain resulted in loss of expression of the mutated protein, suggesting that this amino acid may be critical for initial protein

folding and stabilization after protein translation (unpublished results).

In this study, we used peptide phage display to map functional domain(s) on CD47 defined by an inhibitory anti-CD47 mAb C5D5 that inhibits PMN transmigration and blocks CD47 binding to SIRP α . Although both linear and constrained phage display libraries were used, all C5D5-binding sequences were identified from the structurally constrained libraries (CL10, CL6). Our previous protein chemistry studies indicated that mAb C5D5 only recognizes nondenatured and nonreduced CD47 (data not shown), it is likely that the Ab epitope is dependent on protein folding and not represented in the linear polypeptide sequence. Our panning results with cysteine-constrained phage libraries and resultant structurally constrained binding peptide sequences support this view. Analysis of C5D5-binding sequences in Table I reveals a prominent feature of the presence of a GW doublet as well as several other consensus residues. We further found the representative peptide CERVIGTGWVRC (peptide C5D5.1) to be the highest affinity Ab binding sequence and provide experimental evidence that this sequence mimics the Ab epitope on CD47. In addition, we show that the peptide C5D5.1 effectively competes with CD47 for binding with its ligand SIRP α . Structural studies using Ig loop(s)-specific SIRP α and Fc fusion proteins further demonstrated that C5D5.1 directly binds to the same Ig loop on

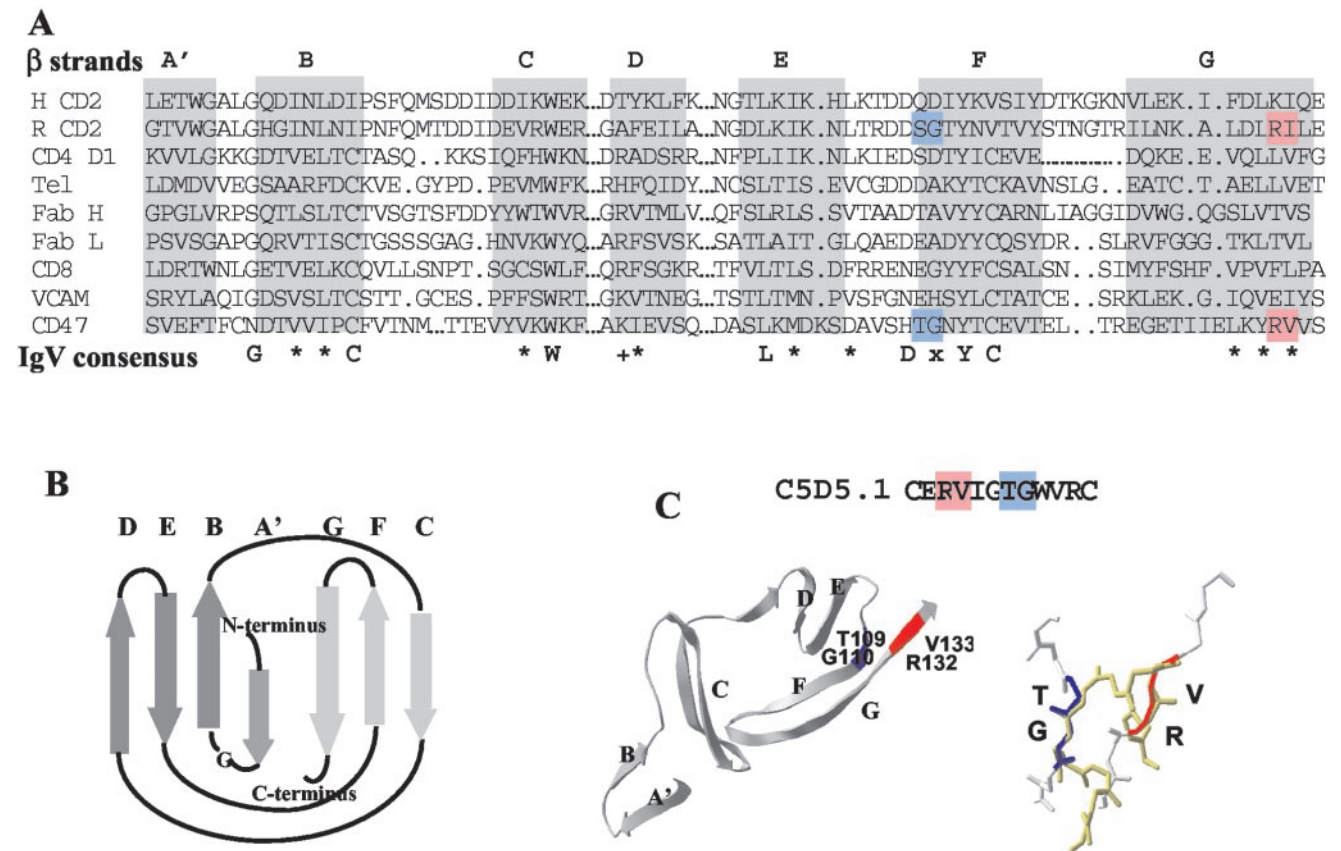


FIGURE 5. A putative SIRP α binding domain on the CD47 protein surface. *A*, The CD47 IgV domain sequence is aligned with other IgV superfamily members based on consensus primary sequence patterns (10): +, basic amino acids; *, hydrophobic amino acids. Structurally conserved β strands (A' to G) are shaded. *B*, The conserved “Greek Key” topology of IgV structure (10) containing two layers of antiparallel β -strand sheets. Note that the sheet containing strands G, F, and C contains residues on the protein surface. *C*, Protein modeling of peptide C5D5.1. *Left*, Modeling of CD47 extracellular IgV domain based on the IgV structure of CD2 (ribbon model) (39). The putative binding site containing residues RV and TG is colored (red and blue, respectively) and illustrates the proximity of these residues on the protein surface. *Right*, Overlaying the entire C5D5.1 sequence (gold) onto residues TG (blue) and RV (red) on F and G strands. Protein structural modeling was performed using The Deep View/Swiss-PDB Viewer version 3.7 (GlaxoSmith-Kline, Research Triangle Park, NC).

SIRP α extracellular domain as that of CD47, thus suggesting that the peptide sequence CERVIGTGWVRC likely mimics at least part of the functional protein surface domain on CD47 that mediates CD47-SIRP α binding. This interpretation is supported by the potent inhibitory effects of mAb C5D5 on CD47-SIRP α binding. Given that the three dimensional structures of CD47, SIRP α , and their binding interactions are not known, our peptide sequence data provides the first clues as to which residues on the surface of CD47 may be involved in binding to SIRP α .

Despite a lack of crystal structure data for CD47, information from other characterized Ig superfamily members can be used to obtain clues as to the localization of C5D5.1 peptide motif on CD47. As highlighted in the sequence alignments shown in Fig. 5, the CD47 extracellular domain shares characteristic sequence criteria similar to other IgV members (10, 31). Thus, it is reasonable to assume that the CD47 extracellular domain structurally forms a similar modular architecture as Ig and other IgV proteins. As shown in Fig. 5, crystal structures of a number of IgV members share a common topology containing two layers of antiparallel β -strand sheets with one layer comprising strands D, E, B, and the N-terminal portion of A, while the other layer comprising G, F, and C strands and the C-terminal end of the A strand (A') (32, 33). Structural analysis using site-directed mutagenesis have revealed that the homophilic and heterophilic adhesive functions of IgV domains are localized to the protein surface that contains G, F, and C strands (34–38). Using the IgV domain structure of CD2 as template, we overlaid the CD47 extracellular IgV sequence and looked for similarities to the core sequence of C5D5.1 (ERVIGTGWVRC) on the putative G, F, and C strands. Two adjacent regions on the G and F β -strands were identified and highlighted in Fig. 5. As shown on the ribbon structure, G strand residues R132, V133, and F strand residues T109, G110 lie in close proximity all within distances of 3–6 Å (N-N distance). A model of peptide C5D5.1 (gold) can be overlaid on CD47 residues TG (blue) and RV (red) with acceptable bond lengths and energy (stick model). Thus, with this model based on well-characterized structure of other IgV members, it is possible to recapitulate the core peptide sequence of C5D5.1 on a predicted, exposed surface on the IgV loop of CD47. In addition, we predict that the single Trp residue on peptide C5D5.1 is not likely to be represented by C strand W58, the most conserved residue within IgV domains, because this Trp is distant and is predicted to project inwards toward the hydrophobic core of the protein. The only other candidate Trp residue is W136 that follows the F strand and lies outside of the Ig structural motif. Although this Trp cannot be modeled in the same way, W136 is a good candidate member of the functional epitope with close proximity to R132, V133. Studies with purified recombinant proteins mutated at these sites will help to clarify this.

Our previous studies demonstrated that CD47-SIRP α interactions regulate PMN transmigration (15). In this study, we have shown that the cyclic peptide C5D5.1 mimics the functional epitope on CD47 involved in both binding to SIRP α and regulating PMN transmigration. Because PMN transepithelial migration is enhanced by epithelial transfection with CD47 (5), it is likely that *trans* interactions between SIRP α on PMN and tissue-expressed CD47 serve to positively regulate PMN migration. This may seem at odds with the inhibitory effects we observe with soluble CD47-AP (15) and peptide C5D5.1. However, since PMN coexpress multiple SIRPs and CD47, it is also likely that *cis*-interactions between SIRPs and CD47 occur within the PMN membrane during activation and contribute to functional regulation. Further characterization of these complex interactions between SIRP and CD47 will shed new light on the regulation of neutrophil migration through tissues during host defense.

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