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Mechanism of CD47-induced $\alpha_4\beta_1$ Integrin Activation and Adhesion in Sickle Reticulocytes*

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We recently reported that CD47 (integrin-associated protein) on sickle red blood cells (SS RBCs) activates G-protein-dependent signaling, which promotes cell adhesion to immobilized thrombospondin (TSP) under relevant shear stress. These data suggested that signal transduction in SS RBCs may contribute to the vaso-occlusive pathology observed in sickle cell disease. However, the CD47-activated SS RBC adhesion receptor(s) that mediated adhesion to immobilized TSP remained unknown. Here we demonstrate that the $\alpha_4\beta_1$ integrin (VLA-4) is the receptor that mediates CD47-stimulated SS RBC adhesion to immobilized TSP. This adhesion requires both the N-terminal heparin-binding domain and the RGD site of TSP. CD47 signaling induces an "inside-out" activation of $\alpha_4\beta_1$ on SS RBCs as indicated by an RGD-*dependent* interaction of this integrin with soluble, plasma fibronectin. However, CD47 engagement also induces an $\alpha_4\beta_1$-mediated, RGD-*independent* adhesion of SS RBCs to immobilized vascular cell adhesion molecule-1 (VCAM-1). CD47 signaling in SS RBCs appears to be independent of large scale changes in cAMP formation but nonetheless promotes $\alpha_4\beta_1$-mediated adhesion via a protein kinase A-dependent, serine phosphorylation of the α_4 cytoplasmic domain. CD47-activated SS RBC adhesion absolutely requires the Src family tyrosine kinases and is also enhanced by treatment of SS RBCs with low concentrations of cytochalasin D, which may release $\alpha_4\beta_1$ from cytoskeletal restraints. In addition, CD47 co-immunoprecipitates with $\alpha_4\beta_1$ in a sickle reticulocyte-enriched fraction of SS RBCs. These studies therefore identify the $\alpha_4\beta_1$ integrin on SS RBCs as a CD47-activated receptor for TSP, VCAM-1, and plasma fibronectin, revealing novel binding characteristics of this integrin.

The $\alpha_4\beta_1$ integrin is a recognized cell-surface receptor for the plasma and matrix protein fibronectin (Fn)[1] and the endothelial cell receptor, vascular cell adhesion molecule-1 (VCAM-1) (1); these interactions are widely assumed to play an important role in leukocyte physiology (2) and blood cell maturation (3–5). Unlike many integrins, $\alpha_4\beta_1$ binds to these ligands in an RGD-*independent* manner (6–8). However, this integrin can also interact with Fn in an RGD-*dependent* manner (9).

The $\alpha_4\beta_1$ integrin is suspected to assume multiple "activation" states (10, 11). Consistent with our expanding knowledge of integrin physiology, the activation state of $\alpha_4\beta_1$ may be regulated by several factors, including divalent cation concentration and agonist-induced "inside-out" cell signaling (12, 13), which could induce the conversion of $\alpha_4\beta_1$ to an active conformation. Recently, the α_4 cytoplasmic domain has been demonstrated to be directly phosphorylated *in vitro* by cAMP-dependent protein kinase A (PKA) (14), suggesting that activation of PKA could impact the activation state of $\alpha_4\beta_1$. Activation of $\alpha_4\beta_1$, as with other integrins, may also confer the ability of this integrin to bind additional ligands. For instance, although $\alpha_4\beta_1$ is not a well characterized thrombospondin (TSP) receptor, an activated form of $\alpha_4\beta_1$ has been reported to bind TSP in the N-terminal heparin binding region of TSP (15, 16). However, a requirement for the RGD sequence in any mechanism of active $\alpha_4\beta_1$ binding to any ligand has never been demonstrated.

Regulation of $\alpha_4\beta_1$ activation *in vivo* may promote leukocyte adhesion (2). Likewise, a pathological role for this integrin receptor is being realized in SS RBC adhesion in sickle cell disease (SCD) (17, 18). SS RBCs are typically two to ten times more adherent than normal RBCs (19, 20) and may impair blood flow in SCD by adhering to factors that line the blood vessel wall or circulate in plasma (18). SS RBC adhesion to the $\alpha_4\beta_1$ ligands TSP, VCAM-1, and Fn or other factors in the vasculature may dramatically impact vaso-occlusion. Either soluble TSP, which is abnormally elevated in the plasma of SCD patients (21), or soluble Fn may serve as a "tether" to anchor circulating SS RBCs to vascular endothelium (22, 23), monocytes (24), and platelets (25). TSP is also present in the subendothelial matrix and is exposed to flowing blood at sites of vascular injury. This exposed TSP potentially serves to anchor SS RBCs to the vascular wall. The resulting adhesion of SS RBCs to the endothelium and/or vascular proteins in the blood vessel is likely to slow or stop blood flow. Furthermore, the *in vitro* adhesivity of an SCD patient's cells has been positively correlated to the severity of their disease (26).

In an effort to better understand the mechanism(s) of SS RBC interaction with the matrix and plasma molecule TSP, we

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[1] The abbreviations used are: Fn, fibronectin; VCAM-1, vascular cell adhesion molecule-1; PKA, protein kinase A; PKAI, PKA inhibitor; PTX, pertussis toxin; TSP, thrombospondin; SCD, sickle cell disease; SS RBCs, sickle red blood cells; mAb, monoclonal antibody; AA, normal hemoglobin; SS, sickle hemoglobin.

recently identified CD47, a known receptor for soluble TSP, as a potent signaling receptor on SS RBCs that upon engagement by *soluble* TSP, activates intracellular signaling events, resulting in SS RBC adhesion to *immobilized* TSP markedly above basal levels (27). These results pointed to a novel mechanism of activated, agonist-induced SS RBC adhesion to TSP and potentially other vascular proteins. However, the CD47-activated adhesion receptor on SS RBCs that mediates this stimulated adhesion to immobilized TSP remained unknown.

Classically, CD47 associates with integrins and activates integrin-dependent adhesion in other hematopoietic cells (28). In platelets, CD47 co-immunoprecipitates with and activates both integrin $\alpha_{IIb}\beta_3$ (29) and $\alpha_2\beta_1$ (30). In other cell lines, CD47 activates integrins $\alpha_v\beta_3$ and $\alpha_4\beta_1$ (15, 31). Although circulating mature erythrocytes have lost integrin expression, we and others have shown that $\alpha_4\beta_1$ expression persists and is the *only* integrin present on circulating immature sickle RBCs (32, 33). Thus, SS RBCs provide an excellent model to unravel $\alpha_4\beta_1$ biochemistry, as these cells allow for the isolated study of this integrin in an abundant, pathophysiologically relevant system.

We have demonstrated that the reticulocyte population of circulating SS RBCs is the most responsive to CD47-stimulated adhesion to TSP (27). Other studies have demonstrated that increased reticulocyte count correlates with SCD severity (34); therefore, the combination of increased soluble TSP levels, reticulocytosis, and the capacity of CD47 to initiate signaling in SS RBCs could provide a potent mechanism through which SS RBC adhesion could promote vaso-occlusion *in vivo*.

Given the unrealized binding and activation potential of $\alpha_4\beta_1$ on SS RBCs, we asked whether this integrin was the unknown receptor for immobilized TSP. We now show that $\alpha_4\beta_1$ is the TSP receptor that becomes activated upon CD47-induced stimulation of SS RBCs. Furthermore, the CD47-activated $\alpha_4\beta_1$ on these cells exhibits an unusual combination of adhesive properties by requiring both the N-terminal domain of TSP as well as the more C-terminal RGD site in this molecule. CD47-activated $\alpha_4\beta_1$ on SS RBCs also recognizes soluble Fn in an RGD-dependent manner. However, CD47 signaling promotes SS RBC adhesion to immobilized VCAM-1 in an RGD-independent manner. Furthermore, we demonstrate that CD47 and $\alpha_4\beta_1$ are complexed on the surface of SS RBCs and that efficient $\alpha_4\beta_1$-mediated adhesion, as with several other integrins (35, 36), requires cytoskeletal remodeling. We have elucidated part of the mechanism of CD47-induced, $\alpha_4\beta_1$-mediated adhesion and integrin activation in these cells and show that CD47-mediated activation of $\alpha_4\beta_1$ requires a PKA-dependent phosphorylation of the α_4 cytoplasmic domain that occurs without a measurable increase in cAMP above basal levels. The activation of $\alpha_4\beta_1$ is also dependent on the activity of the large G-protein G_i, but may be mediated by G_i activation of the Src family of tyrosine kinases rather than by a direct coupling of G_i to adenylyl cyclase. Taken together, our results further reinforce the importance of $\alpha_4\beta_1$ as a therapeutic target in SCD.

EXPERIMENTAL PROCEDURES

Reagents—Human purified recombinant VCAM-1 was obtained from R&D Biosciences (Raleigh, NC), purified platelet TSP was from Sigma, and purified plasma Fn was from Invitrogen. The anti-CD47 monoclonal antibody, IF7, was a generous gift from Dr. Eric Brown, University of California, San Francisco (37). The hybridoma for B6H12 was obtained from ATCC, and B6H12 was purified from cell culture supernatant by an Immunopure® (G) IgG purification kit (Pierce). Antibodies against the α_4 and β_1 integrin subunits and Fn were obtained from Chemicon (Temecula, CA). The conformationally specific antibodies against TSP were obtained as follows. The M1A antibody was a generous gift from Jack Lawler (Brigham and Women's Hospital and Harvard Medical School), and the D4.6 antibody against TSP was purchased from Labvision (Fremont, CA). The monoclonal antibody against TSP, C6.7, was purchased from Labvision. TSP peptides 4N1K (kRFYVVMWKk) and control 4NGG (kRFYGGMWKk) were synthesized and high pressure liquid chromatography-purified by the University of North Carolina (UNC)-Chapel Hill Protein Chemistry Laboratory. In this work, either RGDS (the native sequence in Fn), RGDA (the native sequence in TSP), or RGDW peptides were used interchangeably and were purchased from Bachem (Torrance, CA) or synthesized at the University of North Carolina as described above. The results from either of the RGD peptides were the same. Okadaic acid, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, cytochalasin D, PP2, forskolin, PKAI were obtained from Calbiochem. The NoC1 peptide was a generous gift from Dr. Dean Mosher, University of Wisconsin at Madison. The α-PS α_4 monoclonal antibody was provided by co-author Dr. J. Han. Unless otherwise indicated, all other reagents were obtained from Sigma.

RBC Preparation—Sickle RBCs were obtained from homozygous sickle cell patients with informed consent during routine clinic visits to the University of North Carolina Comprehensive Sickle Cell Center. All studies with human subjects followed the guidelines of the UNC-Chapel Hill institutional review board and the tenets of the Helsinki protocol. All blood samples were drawn by venipuncture into 0.13 M sodium citrate and processed immediately by centrifugation at $150 \times g$ for 15 min at ambient temperature to isolate RBCs from plasma and platelets. RBCs were then washed three times in CGS (1.29 mM sodium citrate, 3.33 mM glucose, 124 mM NaCl, pH = 7.2). For flow adhesion assays, a 1% hematocrit was then prepared by diluting 30 μl of packed RBCs in 1.5 ml of Hanks' balanced salt solution (Invitrogen) supplemented with 0.3% bovine serum albumin (Serologicals, Kankakee, IL) and 20 mM HEPES, pH 7.4.

Flow Adhesion Assay and Protein Immobilization—The flow adhesion system used for this study was designed to mimic blood flow through postcapillary venules as described previously (38). Briefly, purified TSP (18.75 ng/ml), VCAM-1 (50 μg/ml), or the NoC1 peptide (50 μg/ml) in perfusion medium was immobilized onto identical wells formed by a silicon gasket in a 35-mm polystyrene culture dish by incubating for 2 h at 37 °C. A 1% hematocrit solution (1.5 ml) was flowed over the wells at a rate of 1.0 ml/min and a constant shear stress of 1 dyne/cm^2. After a wash period of ~4 min, the number of adherent cells from four representative areas of the well were counted using direct light microscopy. The counted cells were then averaged and presented as adherent cells/mm^2.

Density Fractionation of SS RBCs—Reticulocyte-enriched fractions of SS RBCs were prepared by centrifuging washed SS RBCs over an arabinogalactan gradient (Larex, St. Paul, MN) ($74{,}000 \times g$, 20 °C, 45 min). SS RBCs collected from low density fractions were resuspended in perfusion medium and packed at $400 \times g$ for 10 min at ambient temperature. Cells were resuspended in lysis buffer as described below.

RBC Lysis and Western Blotting—For co-immunoprecipitation studies, fractionated or unfractionated RBCs were lysed for 20 min in ice-cold lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 137 mM NaCl, 500 μM EGTA, 100 mM $NaVO_3$, 100 mM NaF, and 1× Protease Inhibitor Cocktail Set III (Calbiochem). The Triton X-100-soluble portion from both samples was incubated overnight at 4 °C with either an anti-CD47 or anti-β_1 integrin at 2 μg/ml final concentration followed by incubation for 1 h with protein G-agarose beads (Pierce). Immunoprecipitated proteins were washed three times in lysis buffer, loaded on 4–20% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blotted with an anti-CD47 mAb, B6H12. For α_4 cytoplasmic domain phosphorylation studies, washed SS RBCs were pretreated with 19 ng/ml okadaic acid (37 °C, 120 min), lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.05% Tween 20, 2 μg/ml aprotinin, 40 μg/ml bestatin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.5 mM Pefabloc, 20 mM glycerophosphate, 50 μM sodium vanadate, 1 mM NaF, 10 mM *p*-nitrophenol phosphate) (39) and incubated as before with an anti-α_4 antibody from Chemicon (20 μg/ml) followed by SDS-PAGE and transfer to polyvinylidene difluoride membranes. Membranes were then Western blotted with α-PSα_4, a monoclonal antibody raised against the Ser^{988}-phosphorylated form of the cytoplasmic domain of the α_4 integrin (14).

Determination of cAMP Levels in SS RBCs—cAMP levels in SS RBCs were determined exactly as described previously (40).

Conformational Studies with Immobilized TSP—To determine whether the RGD site in TSP is exposed upon protein immobilization, 18.75 ng/ml TSP was allowed to adhere for 2 h at 37 °C in Tris-buffered saline supplemented with 2 mM Ca^{2+} in microtiter wells (Falcon). The wells were washed and then either reduced with β-mercaptoethanol for 1 h at room temperature or maintained in the Ca^{2+}-supplemented Tris-buffered saline. The wells were subjected to enzyme-linked immu-

nosorbent assay using either the D4.6 antibody or the MA1 antibody at a final concentration of 1 μg/ml followed by anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences, San Francisco, CA). The samples were then developed using TMB substrate (3,3′,5,5′-tetramethylbenzidine) and read by Spectra Max Plus (Molecular Devices, Sunnyvale, CA) microplate reader.

Immunoprecipitation of TSP from SS Patient Plasma—Sickle cell patient samples were drawn into D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone anticoagulant to maintain Ca^{2+} levels, and platelet poor-plasma was obtained by immediate centrifugation at $150 \times g$ to separate platelet-rich plasma from red blood cells and white blood cells. The platelet-rich plasma was then incubated with 40 ng/ml prostaglandin I_2 to prevent platelet activation and release and then centrifuged at $200 \times g$ to remove platelets. The TSP antibody D4.6 or C6.7 (1 μg/ml final concentration) was then added to the platelet-poor plasma and incubated for 2 h at room temperature followed by the addition of 30 μl of a 50% slurry of protein G-agarose (Amersham Biosciences). The samples were then incubated for an additional hour at room temperature and centrifuged. Immunoprecipitated proteins were then washed in Tris-buffered saline supplemented with 2 mM Ca^{2+}, reduced, and subjected to SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membranes and Western blotted with the C6.7 antibody against TSP.

Determination of Soluble Fn Binding—Freshly drawn, washed SS RBCs were stimulated with 50 μM 4N1K agonist peptide or 4NGG control peptide for 30 min at 37 °C. The red blood cells were then washed by centrifugation to remove residual agonist and incubated with 200 μg/ml human plasma Fn (41, 42) for 30 min at 37 °C, washed again, and incubated with an anti-Fn monoclonal antibody for 15 min at room temperature. The cells were then washed and incubated briefly with an anti-mouse R-phycoerythrin-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). The cells were washed again and analyzed by flow cytometry.

Statistics—The significance of any differences for all treatments studied was determined using a two-tailed Student's t test. Unless otherwise noted, $p < 0.001$.

RESULTS

We reported previously that stimulation of CD47 on SS RBCs induces their adhesion to immobilized TSP. However, the adhesion receptor(s) on SS RBCs that mediated this induced adhesion remained unknown. The $\alpha_4\beta_1$ integrin is expressed on sickle reticulocytes (33) and is thought to partially mediate basal sickle reticulocyte adhesion to vascular endothelial cells via an interaction with VCAM-1 expressed on the endothelial cell (43). Because CD47 had been demonstrated to stimulate $\alpha_4\beta_1$ function in certain T-cell lines (15), we asked whether this integrin mediated CD47-induced adhesion to immobilized TSP using SS RBCs freshly isolated from SCD patients. We therefore induced SS RBC adhesion to immobilized TSP by treating the SS RBCs with activating ligands for CD47, either soluble TSP or the 4N1K agonist peptide (44), and evaluated stimulated SS RBC adhesion under shear conditions in a flow adhesion assay. Soluble TSP in the presence of an isotype-matched control IgG induced a 3-fold increase in SS RBC adhesion to immobilized TSP. Preincubation with either an α_4-blocking mAb or a β_1-blocking mAb alone completely inhibited the CD47-stimulated portion of SS RBC adhesion to immobilized TSP (Fig. 1*a*). Incubation with submaximal concentrations of the α_4- and β_1-blocking mAbs in combination also completely blocked TSP-stimulated adhesion to TSP (Fig. 1*a*). These results suggest that each integrin subunit contributed to the stimulated SS RBC adhesion. However, neither antibody alone or in combination had any effect on basal SS RBC adhesion to TSP (data not shown). Similar results as above were obtained when SS RBCs were stimulated with the 4N1K agonist peptide derived from TSP (data not shown). These data implicate a specific role for $\alpha_4\beta_1$ as the integrin on SS RBCs that mediates CD47-stimulated adhesion to immobilized TSP.

In myeloid cell lines, CD47-stimulated $\alpha_4\beta_1$ binds to the heparin-binding domain at the N terminus of TSP. We therefore evaluated CD47-stimulated SS RBC adhesion to a peptide derived from the N terminus of TSP termed the NoC1 peptide (15). Our data indicate that SS RBCs stimulated with the 4N1K peptide adhered to the NoC1 peptide, and this adhesion could be completely blocked by an α_4 or β_1 function-blocking antibody but not by a control IgG (Fig. 1*b*). However, even at saturating concentrations of immobilized NoC1 peptide (Fig. 1*b*), the degree to which the 4N1K-stimulated SS RBCs adhered was substantially less than the degree to which they adhered to the intact TSP molecule (Fig. 1*a*), suggesting that the NoC1 peptide could not fully recapitulate SS RBC adhesion to intact TSP. In addition, incubation of CD47-stimulated SS RBCs with soluble NoC1 peptide completely blocked stimulated SS RBC adhesion to intact TSP (Fig. 1*a*). These results indicate that CD47-stimulated SS RBCs bind at least in part to the sequence of immobilized TSP defined by the NoC1 peptide. An LDV conjugate, representative of a class of inhibitors known to block $\alpha_4\beta_1$ function, failed to inhibit the $\alpha_4\beta_1$-mediated adhesion to either TSP or the NoC1 peptide (Fig. 1, *a* and *b*, respectively), even though both TSP and NoC1 contain an LDV sequence that has been implicated in $\alpha_4\beta_1$-mediated adhesion to other ligands (45).

Although $\alpha_4\beta_1$ is typically identified as a non-RGD-binding integrin, $\alpha_4\beta_1$ can mediate RGD-dependent interactions with its ligands (9). In addition, $\alpha_4\beta_1$ is typically identified as a receptor for VCAM-1 and Fn but, when activated, can also bind TSP (46). A role for $\alpha_4\beta_1$ as an activated, RGD-dependent receptor for TSP on any cell type had never been evaluated. We therefore asked whether the CD47-induced increase in SS RBC adhesion to immobilized TSP under shear was also RGD-dependent. We found that an RGD (100 μM), but not a control RGE peptide, completely blocked the TSP-stimulated portion of SS RBC adhesion to immobilized TSP (Fig. 2*a*, *left*). Similar results were obtained when SS RBCs were stimulated with the 4N1K agonist peptide (Fig. 2*a*, *right*). Because the RGD peptide could potentially interfere with either soluble TSP or 4N1K binding to CD47, we also stimulated SS RBC adhesion to immobilized TSP with the B6H12 mAb, which in SS RBCs and some other cells activates CD47 signaling (15, 27, 47). Under these conditions, the RGD peptide still blocked SS RBC adhesion to TSP (data not shown). Neither RGD nor RGE had any effect on basal, nonstimulated adhesion to immobilized TSP under shear (data not shown). Unlike the NoC1 peptide, the RGD peptide, when immobilized, failed to support CD47-stimulated SS RBC adhesion (data not shown). However, simultaneous immobilization of NoC1 and the RGD peptide fully reconstituted SS RBC adhesion to the level observed with intact TSP (Fig. 1*b*), further reinforcing a role for the RGD site within TSP in maintaining efficient SS RBC adhesion under shear conditions. However, incubation of the stimulated RBCs with a soluble RGD peptide failed to inhibit the $\alpha_4\beta_1$ mediated adhesion to the NoC1 peptide (Fig. 1*b*) indicating that $\alpha_4\beta_1$ independently binds to both sites in the intact TSP protein under shear conditions. These results suggest that activation of $\alpha_4\beta_1$ on SS RBCs by CD47 induces SS RBC adhesion to immobilized TSP, which requires both the N terminus and RGD-containing regions of immobilized TSP.

Even though immobilized TSP can support cell adhesion via its RGD site (48), several studies have reported that the RGD peptide sequence is cryptic in intact TSP and may require TSP to be either reduced or denatured before the RGD site is available for binding (49). Therefore, we next sought to determine whether the RGD site was accessible to $\alpha_4\beta_1$ in either the immobilized or soluble forms of intact TSP. We first examined the exposure of the RGD site in TSP present in platelet-poor plasma derived from sickle cell patients. It should be noted that plasma levels of TSP are abnormally elevated in SCD (21).

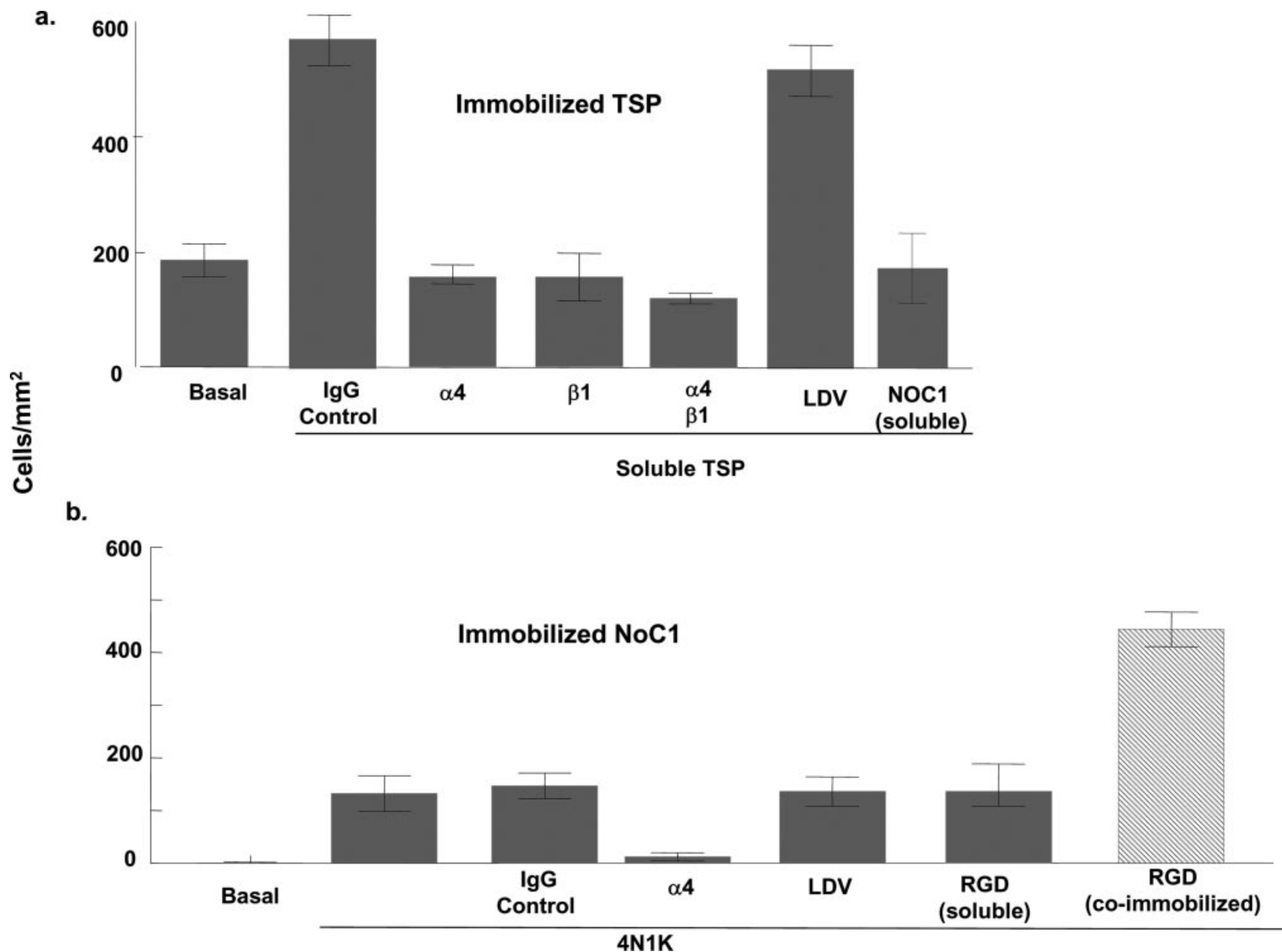

FIG. 1. **CD47-induced SS RBC adhesion to TSP is mediated by $\alpha_4\beta_1$.** *a*, CD47-induced adhesion to TSP is blocked by function-blocking antibodies against the $\alpha_4\beta_1$ integrin. Washed SS RBCs were activated by the addition of 40 nM soluble TSP and allowed to flow over immobilized TSP under physiologically relevant conditions (shear stress, 1 dyne/cm^2; flow rate, 1 ml/min at 37 °C) in the presence of 1 μg/ml isotype-matched control antibody, 1 μg/ml anti-α_4 antibody, 1 μg/ml anti-β_1 antibody, 0.1 μg/ml final concentration of both antibodies together, 1 mM LDV conjugate, or 10 μg/ml soluble NoC1 peptide. *b*, The NoC1 peptide supports CD47-stimulated SS RBC adhesion under shear conditions in an $\alpha_4\beta_1$-dependent manner. Washed SS RBCs were activated by the addition 50 μM 4N1K peptide and allowed to flow over either the NoC1 peptide co-immobilized with the RGD peptide (*striped bar*) or the immobilized NoC1 peptide alone (*solid bars*) in the presence of 1 mM LDV conjugate, 100 μM soluble RGD, or 1 μg/ml α_4 function-blocking antibody or control IgG. Data are shown as the combined mean of 4 patients ± S.D.

Endogenous plasma TSP from these patients was immunoprecipitated with saturating amounts of either the nonconformationally specific antibody C6.7 against the C-terminal CD47-binding domain of TSP (50) or the comformationally specific antibody D4.6, which binds near the RGD-containing region of TSP and has an epitope that is both Ca^{2+}- and redox-sensitive in TSP (51, 52). As shown by Western blot analysis of the immunoprecipitates (Fig. 2*b*), the D4.6 antibody recognized plasma TSP poorly compared with the C6.7 antibody. However, when the platelet-poor plasma was subjected to mild reducing conditions, both mAbs effectively immunoprecipitated equivalent amounts of TSP, suggesting that the D4.6 epitope becomes available on reduced TSP. These results suggest that, in SCD patient plasma, the RGD site near the D4.6 epitope in soluble TSP is relatively cryptic, but the C-terminal portion of TSP, which contains the C6.7 epitope and the CD47 binding site, is exposed and available to bind CD47. We next asked whether immobilization of TSP was sufficient to expose the RGD site in the molecule by adhering reduced and nonreduced TSP to microtiter wells and subjecting samples to enzyme-linked immunosorbent assay using the D4.6 mAb, or MAI, another conformationally specific mAb against TSP (53). Both antibodies bound equally well to reduced or nonreduced immobilized TSP, suggesting that immobilization exposes the RGD site in TSP as efficiently as mild reduction of the molecule (Fig. 2*c*).

To further confirm that $\alpha_4\beta_1$-mediated adhesion was stimulated by CD47 on SS RBCs, we evaluated CD47-induced SS RBC adhesion to a known ligand for $\alpha_4\beta_1$, VCAM-1. Unstimulated SS RBCs adhered poorly to immobilized, purified VCAM-1 under basal shear conditions (Fig. 3*a*). However, both soluble TSP and 4N1K induced marked SS RBC adhesion to immobilized VCAM-1. Furthermore, preincubation of SS RBCs with 1F7, a function-blocking mAb against CD47, but not an isotype-matched control antibody, completely blocked either TSP- or 4N1K-induced SS RBC adhesion to VCAM-1, indicating that CD47 is the agonist receptor responsible for the induced adhesion (Fig. 3*a*). Likewise, this CD47-induced SS RBC adhesion to VCAM-1 was blocked by function-blocking antibodies against α_4 and β_1 either alone (Fig. 3*b*) or in combination (data not shown). CD47-induced adhesion to immobilized VCAM-1, unlike immobilized TSP, was insensitive to RGD inhibition but was completely inhibited by the LDV compound (Fig. 3*b*). Taken together, these results suggest that CD47 induces an increased affinity and/or avidity of $\alpha_4\beta_1$ for VCAM-1 and TSP. However, while CD47 induced an NoC1 and RGD-*dependent* interaction of $\alpha_4\beta_1$ with immobilized TSP, CD47 induced a strictly RGD-*independent* interaction of $\alpha_4\beta_1$ to immobilized VCAM-1 (Fig. 3*b*). Thus, these results suggest that CD47-stimulated $\alpha_4\beta_1$ not only mediates adhesion to several

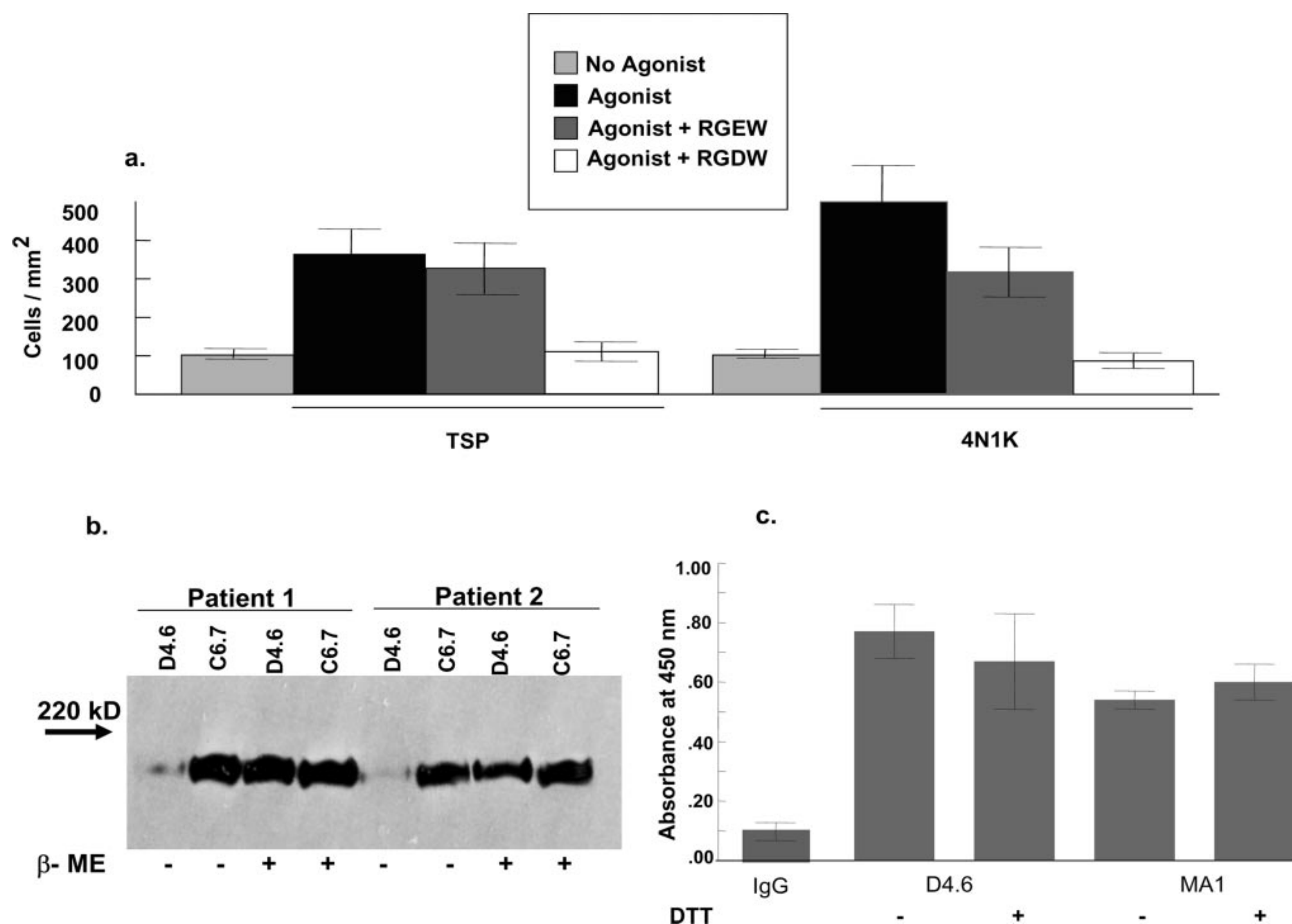

FIG. 2. **RGD site in immobilized TSP is exposed to $\alpha_4\beta_1$ and supports CD47-induced SS RBC adhesion.** *a*, SS RBC adhesion to TSP is blocked by a soluble RGD peptide. Washed SS RBCs were activated with 40 nM soluble TSP (*left*) or 16 μM 4N1K peptide (*right*) followed by incubation with 100 μM RGE control peptide or RGD peptide for 30 additional min at 37 °C and allowed to adhere to immobilized TSP under shear. Data are shown as the combined mean of 4 patient samples ± S.D. *b*, RGD site in SCD plasma is cryptic. SS platelet-poor plasma prepared from two separate patients was left untreated or subjected to mild reduction (0.01 M β-mercaptoethanol (*β-Me*) for 30 min, plasma Ca^{2+} levels), and TSP was immunoprecipitated using the indicated mAbs. Western blots were probed with the C6.7 mAb. Data shown are representative of 4 patient samples. *c*, immobilization of TSP exposes the RGD site. Immobilized TSP was left untreated or reduced (5 mM dithiothreitol (*DTT*) for 2 h, 2 mM Ca^{2+}), and an enzyme-linked immunosorbent assay performed was as described under "Experimental Procedures." Similar results were obtained using 0.01 M β-mercaptoethanol (data not shown). Data shown are representative of 4 independent analyses ± S.D.

ligands relevant to SCD but also recognizes these ligands via distinct mechanisms.

Integrin activation is classically indicated by an increase in soluble ligand binding. By examining the binding of purified, plasma Fn, another RGD-containing ligand, to CD47-stimulated SS RBCs, we tested the hypothesis that $\alpha_4\beta_1$ on SS RBCs is converted to an active state. Soluble plasma Fn is a likely candidate to support tethered SS RBC adhesion to the endothelium in SCD patients (17) and its binding to $\alpha_4\beta_1$ on SS RBCs may help to form the vaso-occlusive blockage in SCD. Incubation with the 4N1K agonist peptide, but not the control 4NGG peptide, dramatically increased soluble Fn binding to the stimulated SS RBCs as detected by flow cytometry (Fig. 4*a*). This increase in Fn binding was completely blocked by preincubating the SS RBCs with the 1F7 antibody, demonstrating the requirement of CD47 signaling (Fig. 4*b*), whereas an isotype-matched control IgG was without effect (data not shown). Furthermore, the increased soluble Fn binding was blocked by either an α_4 or β_1 subunit-specific function-blocking antibody (Fig. 4*c*), demonstrating an $\alpha_4\beta_1$-dependent binding. Isotype-matched control IgGs had no effect on Fn binding in either case (data not shown). Since Fn contains an RGD sequence, we next evaluated whether an RGD peptide would block soluble Fn binding to SS RBCs. Incubation of the CD47-stimulated SS RBCs with an RGD-containing peptide ablated Fn binding, whereas the control RGE peptide was without effect (Fig. 4*d*). The data therefore indicate that CD47-induces inside-out signaling in SS RBCs, which activates $\alpha_4\beta_1$ and renders this integrin capable of binding soluble ligand.

We next sought to examine the inside-out signaling pathway through which CD47 induces $\alpha_4\beta_1$-mediated adhesion in SS RBCs. To this end, we analyzed the phosphorylation state of the α_4 subunit cytoplasmic domain in response to either 4N1K- or TSP-induced CD47 signaling. Phosphorylation of the α_4 cytoplasmic domain has been shown to regulate its interaction with the cytoskeletal protein, paxillin (39), and may affect the adhesive function of $\alpha_4\beta_1$ (12). To detect phosphorylation of the α_4 cytoplasmic domain in SS RBCs, we used α-PSα_4, a well characterized mAb specific for the Ser^{988}-phosphorylated form of the α_4 cytoplasmic domain (14). Although basal levels of α_4 phosphorylation in SS RBCs were not detectable (Fig. 5*a*), both stimulation with the 4N1K peptide and, to a lesser extent, soluble TSP induced phosphorylation of the α_4 cytoplasmic domain in SS RBCs (Fig. 5*a*). B6H12 also induced α_4 cytoplasmic domain phosphorylation, whereas an IgG control antibody was much less efficient (Fig. 5*a*), although similar amounts of the α_4 integrin were present in each lane (Fig. 5*a*, *bottom panel*). These data suggest that CD47 activation promotes serine phosphorylation of this integrin, thus potentially regulating its affinity for ligands.

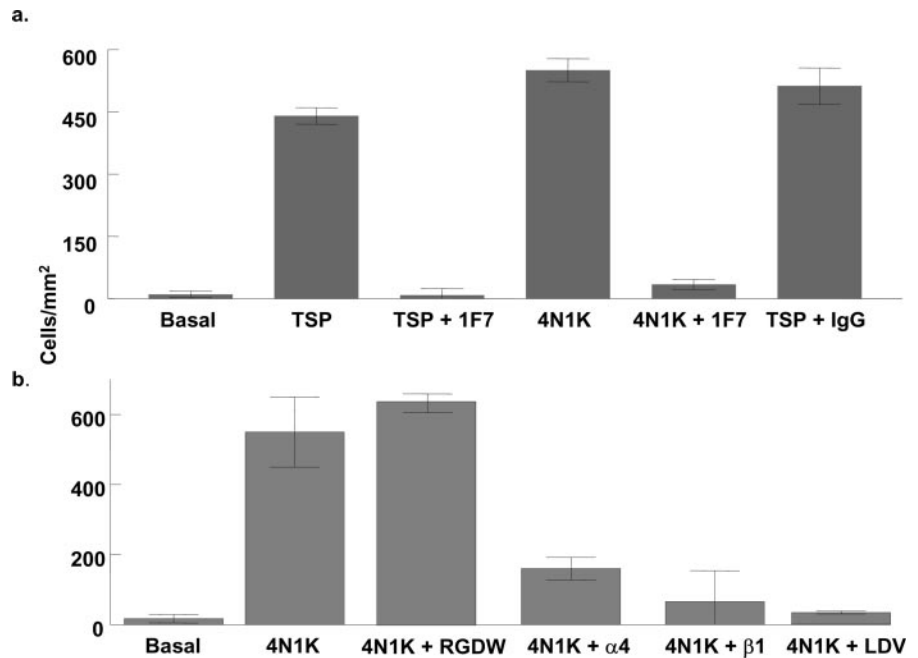

FIG. 3. **Signaling via CD47 induces SS RBC adhesion to VCAM-1 in an $\alpha_4\beta_1$-dependent manner.** *a*, CD47 stimulation induces SS RBC adhesion to immobilized VCAM-1. SS RBCs were treated with 100 nM soluble TSP or 100 μM 4N1K peptide and allowed to flow over immobilized VCAM-1 under shear conditions in the presence or absence of 1 μg/ml CD47 function-blocking antibody 1F7 (30 min pretreatment, 37 °C) or isotype-matched control antibody, and adherent cells were counted (n = 3 separate patients ± S.D.). *b*, CD47-induced SS RBC adhesion to VCAM-1 is $\alpha_4\beta_1$-dependent. SS RBCs were activated by 100 μM 4N1K agonist peptide and allowed to flow over immobilized VCAM-1 in the presence of 1 μg/ml anti-α_4 antibody, 1 μg/ml anti-β_1 antibody, 1 mM RGDW, or 1 mM LDV conjugate. Data are shown as the combined mean of 3 separate patient samples ± S.D.

Previous studies have suggested that phosphorylation of the α_4 cytoplasmic domain occurs within a PKA consensus site (14, 39). We therefore asked whether CD47-induced phosphorylation of the α_4 cytoplasmic domain in SS RBCs was dependent on PKA activity. We found that α_4 phosphorylation was completely blocked by preincubation of SS RBCs with the PKA inhibitor, PKAI (compare Fig. 5*b*, *lanes 2* and *3*).

Interestingly, the CD47-induced α_4 phosphorylation was also blocked by pertussis toxin, a known inhibitor of G_i, which suggests a G_i dependence of this phosphorylation (Fig. 5*b*). This is consistent with the known coupling of CD47 to G_i and the pertussis toxin sensitivity of CD47-induced signaling and SS RBC adhesion (27, 54). However, CD47-induced signaling did not cause a measurable change in overall cAMP levels in these cells compared with a positive control of forskolin treatment (Fig. 5*c*), suggesting that the effects of CD47 signaling occur independently of a rise or fall in cAMP formation in SS RBCs. In some cell membranes, G_i fails to couple with adenylate cyclase in an efficient manner (55). Instead, G_i may preferentially activate Src kinase family members and initiate cell signaling via these enzymes (56–60). Consistent with a role for these tyrosine kinases in CD47-induced G_i-mediated signaling, pertussis toxin completely inhibited CD47-induced tyrosine phosphorylation in SS RBCs (Fig. 5*d*), suggesting that activation of both G_i and these kinases is essential for CD47 signal transduction in SS RBCs. Furthermore, incubation of the 4N1K-activated SS RBCs with PKAI also partially blocked CD47-induced tyrosine phosphorylation, further reinforcing a role for a pathway independent of changes in cAMP levels in SS RBCs.

To correlate CD47-induced α_4 phosphorylation with CD47-induced SS RBC adhesion, we asked whether preincubation of the SS RBCs with PKAI blocked 4N1K-stimulated SS RBC adhesion to immobilized TSP and VCAM-1 under shear conditions. Consistent with a role for PKA in CD47-induced $\alpha_4\beta_1$ phosphorylation, PKAI blocked 4N1K-induced adhesion to both immobilized TSP (Fig. 5*e*) and VCAM-1 (data not shown), suggesting a novel role for PKA as an intermediate in CD47 signaling in SS RBCs. These data indicate a central role for PKA in regulating $\alpha_4\beta_1$-mediated SS RBC adhesion.

We next asked whether the Src family tyrosine kinases played a role in CD47-induced SS RBC adhesion. Preincubation of 4N1K-stimulated SS RBCs with the PP2 Src family kinase inhibitor completely blocked the stimulated portion of SS RBC adhesion to TSP, whereas preincubation with the control PP3 reagent was without effect (Fig. 5*f*). Taken together, these results indicate that Src family kinases are activated downstream of G_i and are required for CD47-induced adhesion to TSP.

To further investigate the mechanism of CD47-induced $\alpha_4\beta_1$-mediated adhesion, we asked whether cytochalasin D, an agent that depolymerizes actin filaments and at low concentrations promotes integrin activation (35, 36), could induce SS RBC adhesion to immobilized TSP. Although incubation of SS RBCs with relatively high concentrations of cytochalasin D had no effect on basal or TSP-induced SS RBC adhesion, low concentrations of this reagent synergized with either 4N1K or soluble TSP to increase SS RBC adhesion to immobilized TSP (Fig. 5*g*) and VCAM-1 (data not shown). However, preincubation of the 4N1K-stimulated SS RBCs with cytochalasin D did not further increase the 4N1K-induced α_4 phosphorylation (Fig. 5*b*). Cytochalasin D alone also caused a modest increase in SS RBC adhesion to TSP and VCAM-1 (data not shown) without inducing detectable basal α_4 phosphorylation (data not shown). Our results with cytochalasin D are also consistent with studies of other integrins, which suggest that integrin activation may result from the release of cytoskeletal restraints (35, 36).

Even though CD47 is known to associate with integrins on other cells, an association of CD47 and $\alpha_4\beta_1$ has never been detected in intact cell membranes. Furthermore, in the RBC membrane, CD47 is a part of the Rh complex of proteins (61), which could potentially exclude or disrupt an integrin/CD47 association. We therefore asked whether we could detect an association of CD47 with $\alpha_4\beta_1$ in either unfractionated or density-fractionated SS RBCs. Unfractionated and unstimulated SS RBCs were lysed and immunoprecipitated for CD47 (Fig. 5*h*, *lane 1*) or the β_1 subunit (Fig. 5*h*, *lane 2*) of the $\alpha_4\beta_1$ complex, and the immunoprecipitates were Western blotted for CD47. CD47 association with β_1 was not apparent in the β_1 immunoprecipitate prepared from unfractionated SS RBCs (Fig. 5*h*, *lane 2*, *top*) or from overestimated amounts of potentially contaminating white blood cells (data not shown). However, in density-fractionated SS RBCs, a co-association of CD47 with β_1 was readily observed in the low density, reticulocyte-

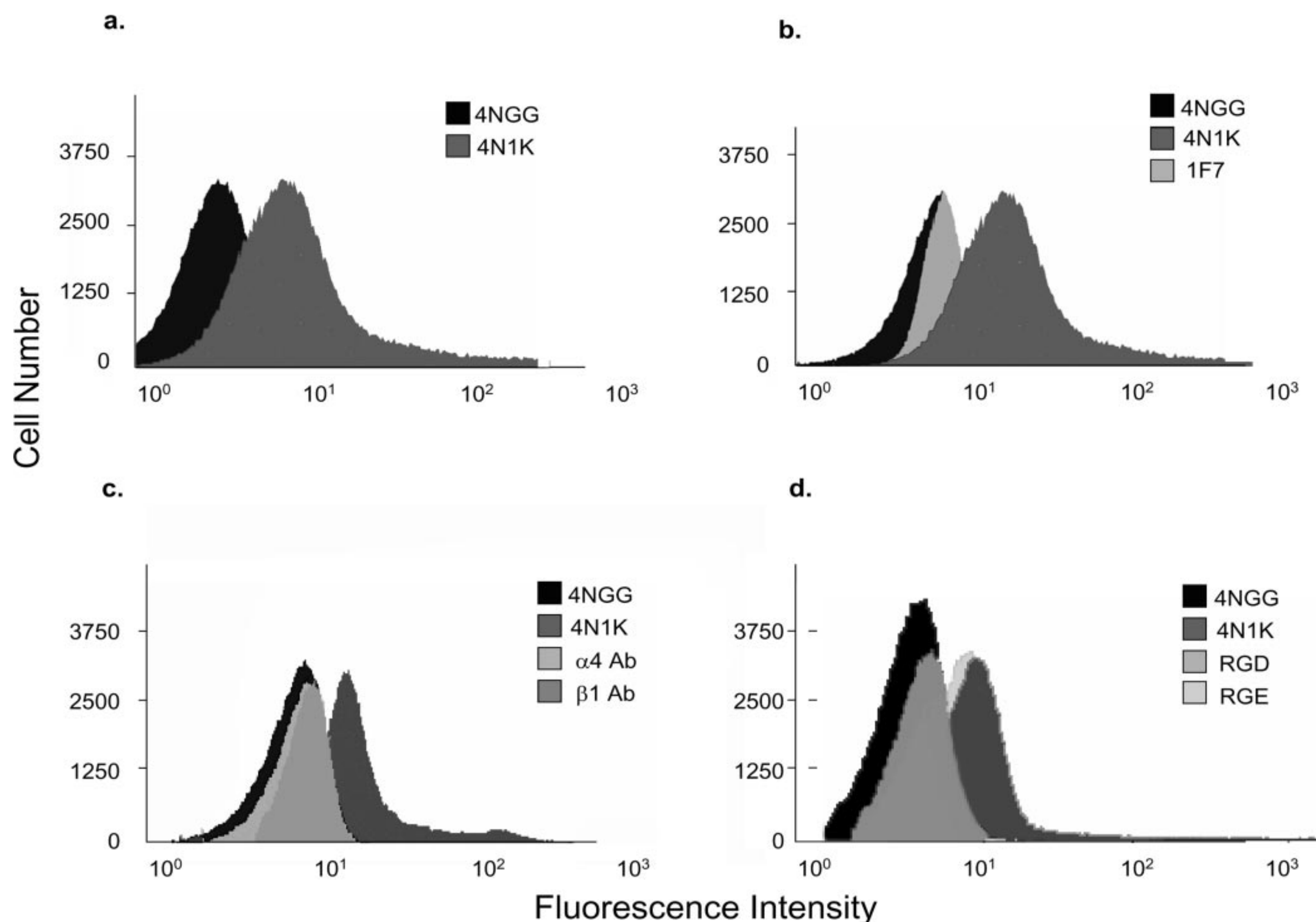

FIG. 4. **CD47 stimulation activates $\alpha_4\beta_1$ on SS RBCs.** *a*, 4N1K peptide promotes soluble Fn binding. Washed SS RBCs were treated as described under "Experimental Procedures" and analyzed for soluble Fn binding via flow cytometry. Data shown are representative of 5 independent patient samples. *b*, CD47 stimulation is required for increased Fn binding. Washed SS RBC were preincubated with 1 μg/ml 1F7 antibody for 30 min at 37 °C prior to incubation with either 4N1K agonist or 4NGG control. Fn binding was then analyzed as described in *a*. Data shown are representative of 3 independent patient samples. *c*, CD47-induced Fn binding is $\alpha_4\beta_1$-dependent. SS RBCs were pretreated with an α_4 or β_1 function-blocking antibody for 30 min at 37 °C prior to exposure to 4NGG or 4N1K. *d*, CD47-induced Fn binding is RGD-dependent. Washed SS RBCs were activated with 16 μM 4N1K peptide followed by incubation with 100 μM RGE control peptide or RGD for 30 additional min at 37 °C and allowed to bind soluble Fn. Data shown are representative of 4 independent patient samples.

enriched fraction (Fig. 5*h*, *lane 2*, *bottom*) but was not observed in isotype-matched control IgG immunoprecipitates (data not shown). These results suggest that CD47 and $\alpha_4\beta_1$ are physically associated with one another in the reticulocyte population of circulating SS RBCs. These results further suggest a strong, detergent-stable interaction between β_1 integrin subunit and CD47 signaling receptor at the SS RBC surface.

DISCUSSION

In this study we show that CD47 stimulates SS RBC binding to immobilized TSP, VCAM-1, and soluble Fn via activation of the $\alpha_4\beta_1$ integrin on these cells. Consistent with other published reports, we now find that CD47-stimulated $\alpha_4\beta_1$ on SS RBCs binds to the N-terminal heparin-binding domain of TSP. However, we also find that efficient CD47-stimulated adhesion to immobilized TSP requires recognition of the RGD site in TSP. Furthermore, we demonstrate that CD47-activated $\alpha_4\beta_1$ on SS RBCs also binds soluble Fn in an RGD-dependent manner. CD47 stimulation also induces an $\alpha_4\beta_1$-dependent adhesion of SS RBCs to VCAM-1, a known ligand for $\alpha_4\beta_1$, via an LDV-sensitive but RGD-insensitive mechanism. We also demonstrate that CD47-induced inside-out signaling results in serine phosphorylation of the α_4 integrin cytoplasmic domain and that this phosphorylation correlates with the ability of SS RBCs to adhere to immobilized TSP and VCAM-1 under physiological conditions *ex vivo*. Our data also indicate that CD47-induced $\alpha_4\beta_1$ activation and adhesion occurs in a PKA-dependent manner that is independent of large scale changes in cAMP levels. Instead, we demonstrate that CD47 signaling in SS RBCs depends on a G_i-mediated activation of the Src family of tyrosine kinases. Finally, we demonstrate that CD47 physically associates with and activates $\alpha_4\beta_1$, most likely by disrupting a cytochalasin D-sensitive interaction with the $\alpha_4\beta_1$ integrin and the erythrocyte cytoskeleton. Our results therefore, suggest a novel role for $\alpha_4\beta_1$ on SS RBCs as an "activable," RGD-binding adhesive mechanism.

One perplexing question from our study is why CD47 appears to function as an agonist receptor only on SS RBCs, even though it is abundantly expressed on RBCs derived from AA individuals (62). Our finding that CD47 couples to $\alpha_4\beta_1$ in SS RBCs may offer an explanation to this phenomenon. In other cells, CD47 is thought to function only in association with integrins; in fact, the CD47-integrin complex may be essential for CD47 signaling (28). Since $\alpha_4\beta_1$ is expressed only on the most immature circulating SS RBCs, *i.e.* reticulocytes, it can be predicted that CD47 should induce an $\alpha_4\beta_1$-dependent adhesion exclusively in these cells, which are extremely rare in the circulation of AA individuals but much more abundant in the blood of SCD patients.

Our data also suggest a role for the N-terminal heparin-binding domain, or the NoC1 peptide of TSP, as a potential site

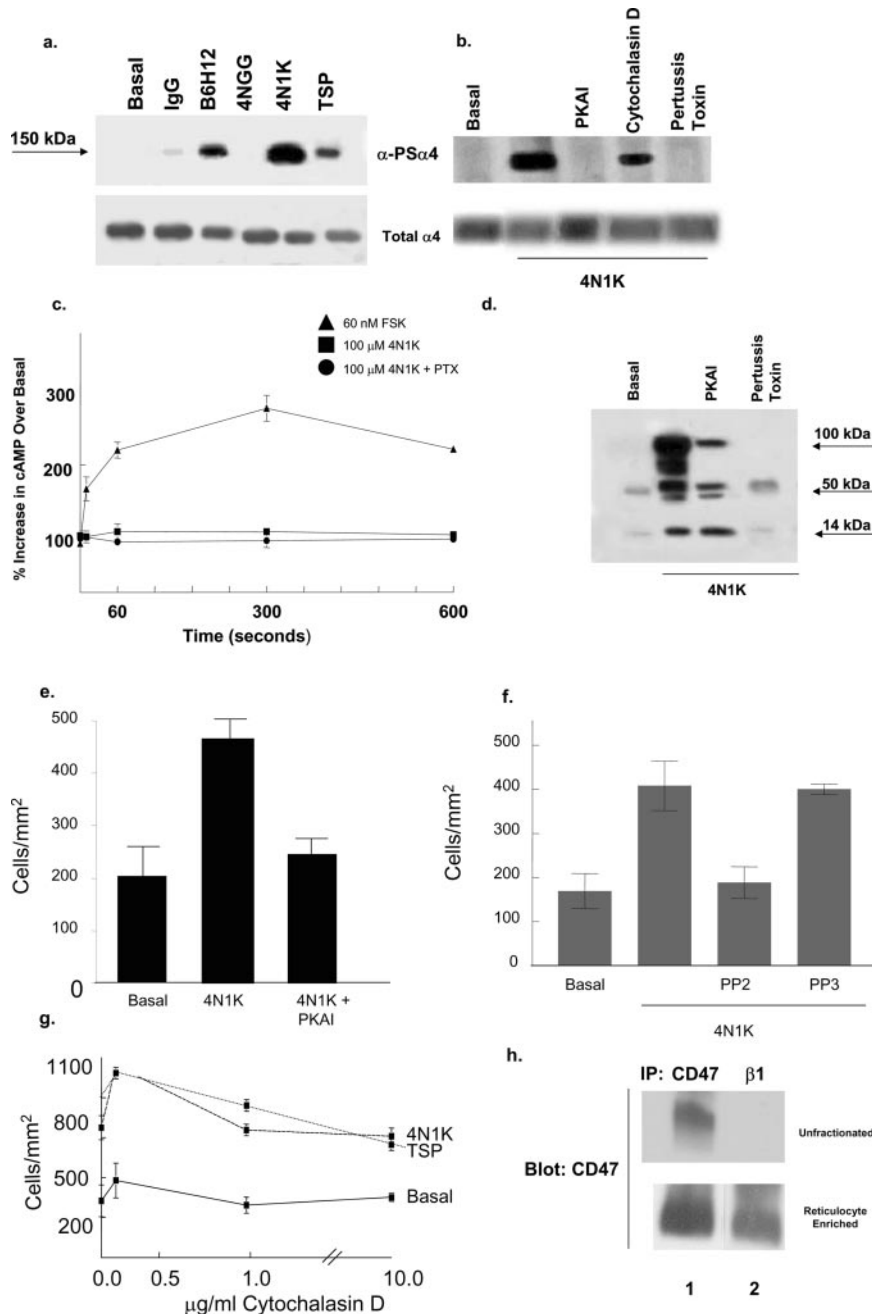

FIG. 5. **CD47 associates with $\alpha_4\beta_1$, induces a G_i- and PKA-dependent phosphorylation of the α_4 cytoplasmic domain, and promotes Src-dependent SS RBC adhesion.** *a*, CD47 induces serine phosphorylation of the α_4 cytoplasmic domain. SS RBCs were unstimulated (*Basal*) or treated with either 1 μg/ml isotype-matched control IgG, 1 μg/ml B6H12, 100 μM 4NGG peptide, 100 μM 4N1K peptide, or 100 nM TSP for 30 min at 37 °C; the phosphorylation state of the α_4 cytoplasmic domain was determined as described under "Experimental Procedures." Total α_4 loaded is shown in the *bottom panel*. *b*, α_4 phosphorylation is PKAI and PTX-sensitive. Treatment of SS RBCs with 36 nM PKAI (2 h, 37 °C), 0.5 μg/ml cytochalasin D (2 h, 37 °C), or 1 μg/ml PTX (30 min, 37 °C) prior to 4N1K stimulation blocked phosphorylation even though equivalent amounts of α_4 were loaded in each lane (*bottom panel*). Blots shown are representative of 3 patient analyses. *c*, treatment of SS RBCs with 4N1K peptide does not elevate cAMP levels in SS RBCs. SS RBCs were treated with either FSK, 4N1K peptide, or PTX (1 μg/ml, 1 h) followed by 4N1K peptide for the times indicated and analyzed for whole cell cAMP content. Data shown are representative of 4 independent patient samples ± S.D. *d*, incubation of 4N1K-stimulated SS RBCs with PKAI or PTX blocks tyrosine phosphorylation. SS RBCs were pretreated with either PKAI or PTX, as described above, followed by 50 μM 4N1K peptide stimulation. The SS RBCs were then analyzed for phosphotyrosine content as described (27). Blot shown is representative of 5 independent patient analyses. *e* and *f*, PKAI or PP2 blocks CD47-stimulated adhesion to TSP in the flow adhesion assay. SS RBCs were incubated as described in *b*, with PKAI or 20 μM PP2 or control PP3, followed by 16 μM 4N1K agonist peptide stimulation and allowed to flow over immobilized TSP. Data shown represent the mean of 2 independent patient samples ± S.D. $p < 0.01$. *g*, low dose cytochalasin D induces SS RBC adhesion immobilized to TSP and VCAM-1. SS RBCs were treated with the indicated concentrations of cytochalasin D only (*solid line, Basal*), 100 μM 4N1K and cytochalasin D (*slashed line*), or 40 nM TSP and cytochalasin D (*dotted line*) and allowed to flow over immobilized TSP. Data represent the average of 4 patient samples ± S.D. *h*, CD47 associates with the β_1 integrin subunit in SS reticulocyte-enriched fractions. CD47 (*lane 1*) or the β_1 integrin subunit (*lane 2*) were immunoprecipitated (*IP*) from unfractionated (*top panel*) or low density reticulocyte-enriched SS RBCs (*bottom panel*) and Western blotted with B6H12. CD47 co-immunoprecipitates with β_1 in low density SS RBCs from density-fractionated preparations (*lane 2*, *bottom panel*) but not unfractionated SS RBCs (*lane 2*, *top panel*). However, similar amounts of CD47 were immunoprecipitated in both fractionated and unfractionated RBCs (*lane 1*). Blot shown is representative of 2 independent patient samples.

to which CD47-stimulated SS RBCs bind. This N-terminal domain, comprising residues 1–356 from intact TSP, also supports Jurkat T-cell binding via activated $\alpha_4\beta_1$ (15).

Although $\alpha_4\beta_1$ typically binds ligands in an RGD-independent manner, this receptor occasionally has been reported to bind substrates in an RGD-dependent manner (9). We found that CD47-stimulated SS RBC binding to both immobilized TSP and soluble Fn was completely blocked by an RGD peptide but not by a control RGE peptide. Since SS RBCs do not express any integrin other than $\alpha_4\beta_1$, this RGD dependence cannot be accounted for by another integrin receptor on these cells. Therefore our data demonstrate for the first time in any cell type a requirement for the RGD site for efficient $\alpha_4\beta_1$-mediated adhesion.

We also observed the activation of SS RBC adhesion by a low concentration of cytochalasin D, which suggests that mild disruption of the erythrocyte cytoskeleton is sufficient to promote adhesivity of these cells. The effects of cytochalasin D on $\alpha_4\beta_1$ activation may mimic PKA-mediated phosphorylation of the α_4 subunit, which disrupts the cytoskeletal attachment of integrin to the cytoskeletal protein, paxillin (39). However, most integrins are known to attach to the cytoskeleton via the cytoplasmic tail of the β subunit (63). Disruption of this interaction with the cytoskeleton has been demonstrated to activate the $\alpha_{IIb}\beta_3$ integrin in platelets (47) and $\alpha_v\beta_3$ in K562 cells (48) presumably by releasing this integrin from its cytoskeletal "restraints," thus allowing a change in conformation to occur. We propose a similar occurrence in SS RBCs and that $\alpha_4\beta_1$ activation in these cells may require contributions from both α subunit phosphorylation and β subunit cytoskeletal linkage.

We also found that the α_4 cytoplasmic domain becomes serine-phosphorylated in response to CD47 signaling in SS RBCs and that this phosphorylation correlates with the ability of SS RBCs to adhere to immobilized TSP and VCAM-1. Phosphorylation of the α_4 cytoplasmic domain in white blood cells plays a potential role in normal white blood cell function and immune response. However agonists that control this phosphorylation had yet to be identified. We now show that α_4 cytoplasmic domain phosphorylation in SS RBCs occurs as a result of physiological agonist stimulation by TSP. Moreover, this phosphorylation may play a role in a devastating illness, sickle cell disease.

Because PTX, an inhibitor of G_i, inhibits CD47-stimulated tyrosine phosphorylation in SS RBCs, α_4 serine phosphorylation, *and* SS RBC adhesion to immobilized ligands, G_i appears to be an early and essential component of the CD47 signaling pathway. Therefore, our study is consistent with several other reports that indicate coupling of CD47 directly to this heterotrimeric G-protein, which is expressed in erythrocytes (64). However, the CD47-induced G_i signaling in SS RBCs appears to function independent of large scale changes in cAMP levels but is dependent on PKA activity. At first, these data seem paradoxical, since α_4 phosphorylation via CD47 signaling requires both G_i, which inhibits of some isoforms of adenylyl cyclase and therefore inhibits cAMP formation, and PKA, a downstream effector of cAMP. However, G_i does not inhibit all isoforms of adenylyl cyclase (55). Since the isoforms of adenylyl cyclase in SS RBCs are not well characterized, these cells may contain adenylyl cyclases resistant to G_i inhibition. Instead, it is likely that G_i may contribute to α_4 phosphorylation and activation of $\alpha_4\beta_1$ via an adenylyl cyclase-independent pathway. Support for this comes from the finding that erythrocytes have Src kinases (65, 66), G_i can stimulate the Src family of tyrosine kinases (56–60), and our finding that CD47-stimulated adhesion in SS RBCs is sensitive to Src kinase inhibition.

A PKA dependence of α_4 phosphorylation and adhesion in the absence of detectable CD47-stimulated cAMP formation may be accounted for in several ways. First, PKA may be localized by AKAPs (protein kinase A-anchoring proteins) (67) and activated by localized pockets of cAMP formation not detected in a whole cell assay. However, AKAP expression or function in erythrocytes has never been examined. Second, several groups have reported cAMP-independent mechanisms of PKA activation (68–70). Third, we previously reported that SS RBCs have significantly elevated basal levels of cAMP (40), suggesting that a cooperation or synergy between basally active PKA and another CD47-stimulated pathway, *e.g.* Src kinases, could result in α_4 cytoplasmic domain phosphorylation and integrin activation. Thus, it is likely that CD47-induced phosphorylation of the α_4 cytoplasmic domain and adhesion rely in part on basally activated PKA, a cAMP-independent PKA activation pathway, and/or localized cAMP formation.

Our finding that CD47 stimulation increases the binding of the soluble $\alpha_4\beta_1$ ligand, Fn, indicates that the inside-out signaling generated via CD47 induces an active conformation of $\alpha_4\beta_1$. Although a CD47-stimulated increase in $\alpha_4\beta_1$ mobility or clustering may also contribute to the increased adhesion, these results suggest a conversion of $\alpha_4\beta_1$ to an active, RGD-binding integrin on SS RBCs and implicate $\alpha_4\beta_1$ as a relevant TSP and Fn receptor. Integrin activation is therefore likely to be a major mechanism mediating the increased adhesion of SS RBCs to selective substrates under shear conditions.

In conclusion, we have demonstrated that the $\alpha_4\beta_1$ integrin in SS RBCs is a target of CD47 signaling and that this integrin appears to require two distinct sites in TSP to support SS RBC adhesion. We have shown both new and established mechanisms of $\alpha_4\beta_1$-mediated adhesion via the N-terminal heparin-binding domain of TSP and an $\alpha_4\beta_1$-mediated, RGD-dependent interaction of these cells with immobilized TSP and soluble Fn. Our results suggest a previously unappreciated mechanism of interaction of this receptor with TSP. Furthermore, we have demonstrated that CD47 signaling induces an RGD-independent adhesion of SS RBCs to VCAM-1 and results in phosphorylation of the integrin α_4 cytoplasmic domain, which appears to be required for $\alpha_4\beta_1$-mediated activation and adhesion to both VCAM-1 and TSP. Modulation of the phosphorylation state of the α_4 cytoplasmic domain and the adhesiveness of SS RBCs to VCAM-1 and TSP in the vasculature has potential therapeutic value in SCD.

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