

The Thrombospondin Receptor Integrin-associated Protein (CD47) Functionally Couples to Heterotrimeric G_i*

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Integrin-associated protein (IAP; CD47) is a thrombospondin receptor that forms a signaling complex with β_3 integrins resulting in enhanced $\alpha_v\beta_3$ -dependent cell spreading and chemotaxis and, in platelets, $\alpha_{IIb}\beta_3$ -dependent spreading and aggregation. These actions of CD47 are all specifically abrogated by pertussis toxin treatment of cells. Here we report that CD47, its β_3 integrin partner, and G_i proteins form a stable, detergent-soluble complex that can be recovered by immunoprecipitation and affinity chromatography. G_{i α} is released from this complex by treatment with GTP or AIF₄. GTP and AIF₄ also reduce the binding of CD47 to its agonist peptide (4N1K) derived from thrombospondin, indicating a direct association of CD47 with G_i. 4N1K peptide causes a rapid decrease in intraplatelet cyclic AMP levels, a G_i-dependent event necessary for aggregation. Finally, 4N1K stimulates the binding of GTP γ ³⁵S to membranes from cells expressing IAP and $\alpha_v\beta_3$. This functional coupling of CD47 to heterotrimeric G proteins provides a mechanistic explanation for the biological effects of CD47 in a wide variety of systems.

The thrombospondins (TSPs)¹ are a family of multidomain, secreted glycoproteins whose production is differentially regulated during development, wound repair, inflammation, tumorigenesis, and other states in which rapid changes occur in cell proliferation and migration (1, 2). Many of the apparently diverse effects of TSP proteins can be ascribed to the presence of different complements of receptors for the several domains of TSP on different cell types. Integrin-associated protein (IAP or CD47) is a receptor for the carboxyl-terminal cell binding domain of TSP1, which contains the active CD47 agonist peptide RFYVVMWK (3, 4). This sequence is well conserved among species and TSP isoforms (2), suggesting that all TSP family members use CD47 as a receptor. CD47 is an unusual member of the IgG superfamily of receptors. It consists of a single IgGv extracellular domain and five transmembrane segments termi-

nating in a short, alternatively spliced cytoplasmic tail (5). Monoclonal antibodies (mAbs) against CD47 block signaling events such as the increase in intracellular Ca²⁺ that occurs when endothelial cells spread on matrix proteins containing RGD sequences (6). Further, anti-CD47 mAbs can block the transendothelial (7) and transepithelial (8) migration of polymorphonuclear nucleophiles, and CD47 knockout mice have a severe host defense defect (9). CD47 appears to be involved in processes related to integrin-mediated cell spreading (4, 10) and motility (3, 11) in a number of cell types.

In several biological settings, the binding to CD47 of TSP1, its cell binding domain, or the agonist peptide 4N1K (KRFYV-VMWKK) can stimulate the "activation" of integrins to a higher affinity/avidity state. For example, the 4N1K peptide stimulates platelet spreading on fibrinogen-coated surfaces and induces aggregation of platelets via activation of the integrin $\alpha_{IIb}\beta_3$ as judged by the enhanced binding of the ligand mimetic mAb PAC-1 (10). When C32 human melanoma cells attach to a sparsely coated vitronectin substratum, TSP1 and 4N1K dramatically stimulate the rate of $\alpha_v\beta_3$ -dependent cell spreading (4). Further, 4N1K peptide is a chemoattractant of endothelial cells migrating on a gelatin/RGD-containing matrix to which they attach via $\alpha_v\beta_3$ (3). The chemotaxis of vascular smooth muscle cells toward soluble collagen is also stimulated by 4N1K. In this case, $\alpha_2\beta_1$, a collagen receptor, is modulated by CD47 (11). In each of these systems, the augmentation of integrin function is selectively blocked by pretreatment of the cells with pertussis toxin, which disables signaling through heterotrimeric G proteins of the G_i family.

The current study was undertaken to determine if the integrin-CD47 complex might functionally associate with a pertussis toxin-sensitive G protein. The data presented here indicate that not only does such an association exist, it has functional consequences for the regulation of GTP binding and cyclic AMP levels. Furthermore, a negative heterotropic effect of GTP γ S and AIF₄ on ligand binding to CD47 suggests that the association of the integrin-CD47 complex with G_i is direct.

EXPERIMENTAL PROCEDURES

Materials—The human melanoma cell line C32 (ATCC CRL 1585) was cultured as described (4), and all reagents were as described by Gao *et al.* (4). Pertussis toxin (List Biologicals) treatment of C32 cells was carried out overnight at 60 and 300 ng/ml media. OV10 cells and stable transfectants derived from them have been described (3, 9). Antibodies against G proteins have the following specificities: 856, G_{i α 1,2,3}; BO87, G_{i α 1} and G_{i α 2}; B600, G _{β 1} and G _{β 2} (12). Peptides were synthesized, purified, and verified by mass spectrometry by the Washington University Protein and Nucleic Acid Chemistry Laboratory. Platelets were obtained from the St. Louis Red Cross or by venipuncture of healthy volunteer donors under institutional guidelines. They were prepared as described by Chung *et al.* (10).

Immunoprecipitations—Crude membrane fractions (100,000 × g pellets) were solubilized with the indicated detergents at 4 °C, and the soluble fraction was incubated with the indicated primary antibody for

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¹ The abbreviations used are: TSP, thrombospondin; IAP, integrin-associated protein; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; NTA, nitrilotriacetic acid; 7TMS, seven-transmembrane segment; GDP β S, guanyl-5'-yl thiophosphate; GTP γ S, guanosine 5'-3-O-(thio)triphosphate.

1–3 h on ice. Appropriate secondary antibodies coupled to agarose were used to collect the bound antigens, and the complexes were washed extensively, solubilized in boiling SDS sample buffer with reduction, and subjected to SDS-PAGE and blotting onto nitrocellulose. Blots were probed with the indicated antibodies as described (4). Affinity labeling in the presence of the indicated compounds with iodinated 4N1K peptide was as described (3, 4).

Affinity Chromatography—His₆-4N1K peptide (1 mg) was adsorbed onto fresh nickel-NTA matrix (200 μ l) and incubated 2 h with a detergent lysate of C32 cell membranes at 4 °C. After washing with 30 ml of HEPES-buffered saline buffer containing the same detergent, stepwise elution with the indicated concentrations of imidazole was initiated (see Ref. 10 for details). After SDS-PAGE, blots were probed with anti- G_α and anti-CD47 antibodies. To determine the amount of CD47 bound to the 4N1K affinity matrix in the presence of various nucleotides, the Triton X-100 (1% w/v) detergent-soluble fraction of C32 membranes was incubated with His₆-4N1K-charged nickel-NTA beads (or His₆-4NGG control beads) for 2 h in the presence of the indicated nucleotides or AlF₄, all at 60 μ M. The beads were rapidly washed and incubated with ¹²⁵I-mAb 2D3 against CD47 for 1 h. After washing, the beads were counted in a Beckman γ -counter. Cyclic AMP in platelets was quantified after ethanol extraction with an enzyme-linked immunosorbent assay kit from Amersham Pharmacia Biotech following the manufacturer's protocol.

GTP γ ³⁵S Binding Assay—K562 cells expressing $\alpha_v\beta_3$ integrin (13) or murine fibroblasts (9) were disrupted by nitrogen cavitation, and membranes were prepared according to Ref. 14. The lysis buffer contained 20 mM HEPES, pH 8.0, 1 mM EDTA, 2 mM MgCl₂, and a protease inhibitor mixture (4). Similar results were obtained when cells were lysed by freeze-thaw cycles. GTP γ ³⁵S (NEG030H; NEN Life Science Products) binding was determined in a filtration assay essentially as described by Sternweis *et al.* (15) in a reaction mixture containing the following: 50 mM HEPES, pH 8.0, 100 mM NaCl, 0.75 mM MgCl₂, 0.1 mM EDTA. Carrier-free GTP γ ³⁵S was present at 3–5 nM final concentration. Binding of GTP γ ³⁵S occurred at 30 °C; reactions, which contained equal amounts of membrane protein (~5–10 μ g), were stopped; and membranes were filtered and washed with ice-cold reaction buffer (15). Membrane protein was determined with a Bradford Assay kit from Bio-Rad.

RESULTS

CD47 and G_i Proteins Co-immunoprecipitate—To investigate the possible association of CD47 and its integrin partner with G proteins, octyl glucoside extracts of C32 cell membranes were affinity-labeled with ¹²⁵I-4N1K peptide (3, 11), resulting in specific labeling of the 52-kDa CD47 (Fig. 1). This labeled lysate was then immunoprecipitated with antibody that recognizes G_α subunits (12). As seen in Fig. 1, the labeled 52-kDa CD47 was recovered in the precipitate (lanes 1 and 6) but not when nonimmune rabbit IgG was used (lane 2). The labeling of the precipitated protein was prevented by excess cold 4N1K peptide during the affinity labeling reaction (lane 3), and no labeled protein was recovered when the ¹²⁵I control peptide 4NGG (KRFYGGMWKK) was used for labeling instead of 4N1K (lane 4). In addition, recovery of labeled CD47 was greatly reduced by preincubation of the anti- G_α antibody with the antigenic peptide (lane 5). In the converse experiment, detergent lysates of C32 cells and human platelets were immunoprecipitated with two different anti-CD47 mAbs (B6H12 and 1F7), and the precipitates were analyzed for the presence of G proteins by Western blotting of SDS gels (Fig. 2A). Both anti-CD47 mAbs coprecipitated 40-kDa G_α and 36-kDa G_β subunits from both cell types. As a further control, OV10 ovarian carcinoma cells, which express no CD47 (3), were used. When these cells are transfected with β_3 integrin cDNA, resulting in expression of $\alpha_v\beta_3$, no G_α protein is recovered in anti-CD47 (1F7) immunoprecipitates (Fig. 2B, lane 3). However, when CD47 cDNA is expressed in these cells, G_α is easily detected in the CD47 complex (lane 1). Coexpression of CD47 along with $\alpha_v\beta_3$ cDNA leads to an increase in the amount of G_α recovered in the anti-CD47 precipitate (lane 2). The amount of G_α expression was comparable in these cell lines (not shown). The magnitude

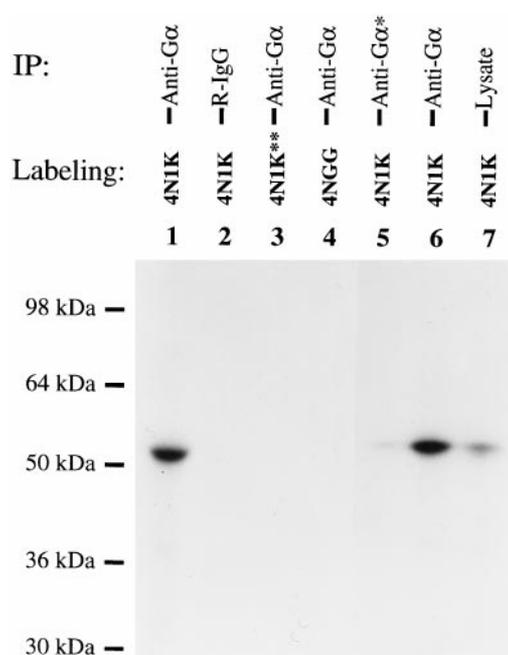


FIG. 1. Immunoprecipitation of affinity-labeled CD47 with anti- G_α antibody. A crude membrane fraction of C32 cells was treated with 30 mM octyl- β -D-glucopyranoside, and the soluble fraction was affinity-labeled with ¹²⁵I-4N1K peptide (lanes 1–3 and 5–7) or ¹²⁵I-4NGG peptide (lane 4) and then immunoprecipitated (IP) with a polyclonal antibody (856) against G_α peptide GAGESGKSTIVKQMK (lanes 1 and 3–6) or rabbit IgG (lane 2). Labeled CD47 in the precipitates was detected by SDS-PAGE and autoradiography. For lane 5, the affinity labeling was performed in the presence of excess unlabeled 4N1K peptide. In lane 5, the anti- G_α antibody was preincubated with the antigenic peptide. Lane 7 is the labeled membrane lysate before immunoprecipitation.

of the increase varies somewhat from one experiment to another ranging from the ~2-fold increase shown in Fig. 2B to nearly 10-fold in some experiments. Thus, a relatively stable complex containing CD47 and heterotrimeric G proteins exists in octyl glucoside extracts of C32 and platelet membranes. The G_α subunits, which complex with CD47 comigrate with purified recombinant G_i standards (Fig. 2) and also react with an antibody (B087; Ref. 12) specific for $G_{i\alpha-1}$ and -2 (not shown). This is consistent with the pertussis toxin inhibition of CD47-dependent signaling responses (4, 10) and earlier reports that chemotactic responses of cells to TSP1 are pertussis toxin-sensitive (16).

Association of G_i and CD47 Is Pertussis Toxin-sensitive—Pertussis toxin catalyzes the ADP-ribosylation of $G_{i\alpha}$ subunits and prevents productive interaction of G_i with receptor (17). C32 cells were treated overnight with either 60 or 300 ng/ml pertussis toxin or its inactive, but surface binding, B oligomer. Membranes were prepared from these cells, and detergent lysates were immunoprecipitated with anti-CD47 mAb 1F7. As seen in Fig. 2C, both concentrations of active toxin cause a decrease in the amount of G protein α - and β -subunits recovered in the CD47 complex. Interestingly, the small amount of G_α remaining in the complex after pertussis toxin treatment migrates at a slightly higher M_r on SDS gels (18), suggesting that ADP-ribosylated G_α retains a marginal affinity for the CD47 complex. Thus, like 7TMS receptors that activate G_i proteins, the signaling from CD47 as well as the association of the G protein is sensitive to pertussis toxin-mediated ADP-ribosylation.

G_i Copurifies with the CD47-Integrin Complex—In an independent approach, we used affinity chromatography to isolate CD47-containing detergent-soluble complexes. 4N1K and

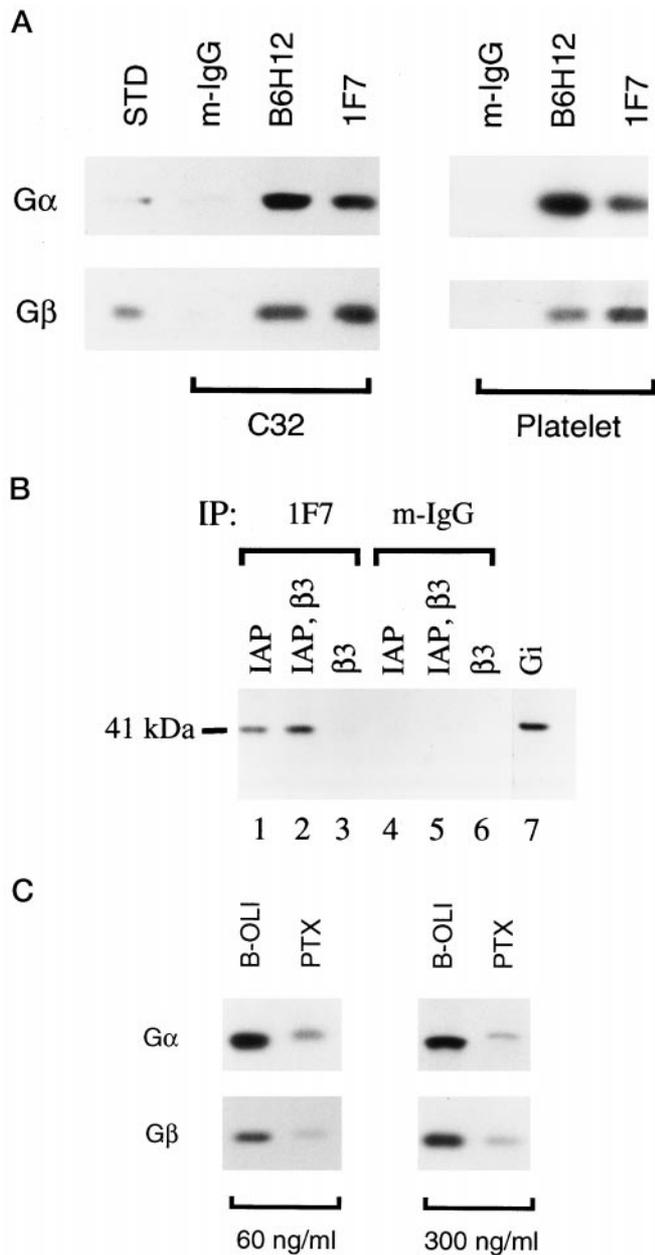


FIG. 2. Immunoprecipitation of G_{α} and G_{β} with anti-CD47 antibodies. A, the octyl- β -D-glucopyranoside (30 mM) soluble fractions of C32 and platelet membranes were immunoprecipitated (IP) with mAb B6H12 or 1F7 against CD47 or mouse IgG control antibody. After SDS-PAGE, blots were probed with antibodies against G_{α} (856, top row) or G_{β} (B600, bottom row) (12). Standard recombinant G protein subunits (prepared as in Ref. 16) are shown in the left lane. B, human ovarian carcinoma cells (clone OV10), which do not express CD47, were transfected with cDNA encoding the β_3 integrin subunit (lanes 3 and 6), CD47 alone (lanes 1 and 4), or CD47 plus the β_3 subunit (lanes 2 and 5) as indicated (13). Detergent-solubilized membranes from these cells were immunoprecipitated with either anti-CD47 mAb 1F7 or control mouse IgG. Recombinant $G_{i\alpha}$ subunit (lane 7) was run on the gel as an internal standard. Expression of β_3 in these cells allows functional reconstitution of $\alpha_v\beta_3$. The levels of endogenous $G_{i\alpha}$ were the same in the three OV10-derived cell lines. C, C32 cells were treated in culture overnight with either 60 or 300 ng/ml *Bordetella pertussis* toxin (PTX) or its inactive B oligomer (B-OLI). Membranes were prepared, solubilized, and immunoprecipitated with anti-CD47 mAb 1F7 as above. The blots were probed with the anti- G_{α} or - G_{β} antibodies.

4NNGG peptides were synthesized with amino-terminal hexahistidine tags and bound to nickel-NTA beads. Detergent lysates of C32 membranes were adsorbed with these affinity beads, washed, and then eluted stepwise with increasing con-

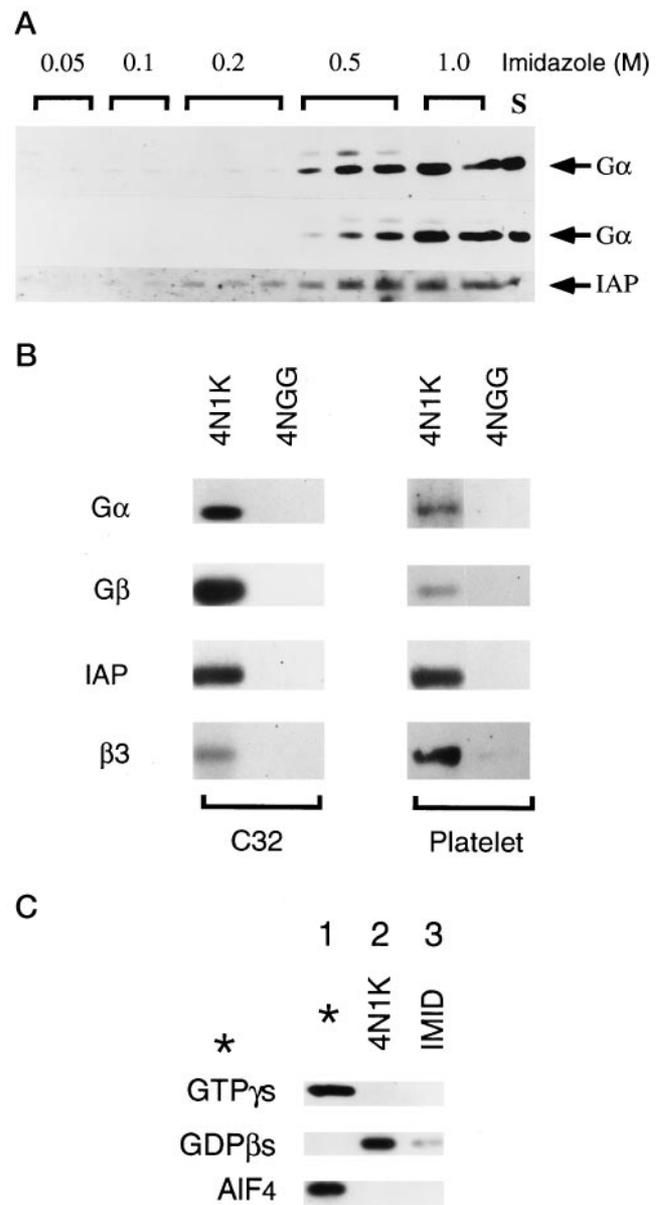


FIG. 3. Affinity chromatography of CD47-G protein complexes. A, G_{α} and CD47 coelute. The detergent-soluble fraction of C32 membranes was applied to a nickel- His_6 -4N1K column as described under "Experimental Procedures." After washing, the column was eluted with the indicated concentrations of imidazole, and the fractions were analyzed by SDS-PAGE and Western blotting with anti- G_{α} and CD47 antibodies. The detergents used were as follows: 30 mM octyl- β -D-glucopyranoside (top row); 1% w/v Triton X-100 (two bottom rows). B, the complex contains G_{α} , G_{β} , CD47, and integrin. The Triton X-100-soluble fraction from C32 membranes or platelets was adsorbed on His_6 -4N1K or His_6 -4NNGG columns, washed with 50 column volumes of HEPES-buffered saline-Triton buffer, and eluted with 1 M imidazole. Fractions of the eluates were run on SDS-PAGE, blotted, and probed with antibodies against G_{α} , G_{β} , CD47, and β_3 integrin. The peak fraction of each eluate is shown. C, $G_{i\alpha}$ is eluted with $\text{GTP}\gamma\text{S}$ and AIF4 but not $\text{GDP}\beta\text{S}$. C32 membrane lysates were adsorbed to His_6 -4N1K affinity columns as above, washed, and eluted with $\text{GTP}\gamma\text{S}$ (30 μM), $\text{GDP}\beta\text{S}$ (30 μM), or AIF4 (30 μM) (lane 1*). Subsequently, each column was eluted with 500 μM 4N1K peptide (lane 2) and then with 1 M imidazole (IMID; lane 3). After SDS-PAGE, the blot was probed with anti- G_{α} .

centrations of imidazole. As seen in Fig. 3A, $G_{i\alpha}$ subunits (solubilized in two different detergents) eluted from a 4N1K column along with CD47 at very high imidazole concentrations (0.5–1.0 M) characteristic of proteins bound via hexahistidine tags. As seen in Fig. 3B, both α and β G protein subunits as well as the β_3 integrin subunit coeluted with CD47 from the 4N1K

column, while none of these were recovered from the control His₆-4NNGG column. To examine the mode of association of the G proteins with CD47 immobilized on the 4N1K column, the lysate-charged column was eluted sequentially with GTP γ S or GDP β S, followed by 4N1K peptide (non-His-tagged) and then imidazole (1 M) (Fig. 3C). GTP γ S quantitatively eluted the $G_{i\alpha}$ protein (and $G_{i\beta}$; not shown) while GDP β S did not, the $G_{i\alpha}$ remaining on the column until elution with 4N1K peptide. In addition, AlF₄ also cleanly eluted the $G_{i\alpha}$ protein (Fig. 3C), indicating that the association of the G proteins with the CD47 complex is released by activation of $G_{i\alpha}$, a property shared with receptor-G protein interactions. Interestingly, GTP γ S and AlF₄, but not GDP β S also eluted about half of the CD47 from the 4N1K affinity column (not shown), suggesting that the conformational change in the $G_{i\alpha}$ subunit associated with activation is translated into a reduced affinity of CD47 for its ligand on the column.

The G_i Activation Affects CD47 Affinity for 4N1K—To investigate more quantitatively this negative heterotropic effect of GTP on the 4N1K-CD47 interaction, the amount of CD47 bound to the 4N1K charged nickel-NTA beads was quantified using an ¹²⁵I-labeled mAb, 2D3, which binds CD47 but does not perturb either ligand binding or signaling from CD47 (4, 10). Detergent lysates of C32 membranes were incubated with the 4N1K-charged nickel-NTA agarose beads along with nucleotides or AlF₄; 4NNGG-charged beads served as a control. After incubation and rapid washing of the beads, the amount of CD47 bound was determined with ¹²⁵I-2D3 mAb. As seen in Fig. 4A, GTP γ S and AlF₄, but not GDP β S or ATP (all at 60 μ M), significantly reduced the amount of CD47 associated with the 4N1K beads. The concentration dependence of the effect of the GTP *versus* the GDP analog is shown in Fig. 4B. The specific inhibitory effect of GTP and AlF₄ on agonist peptide binding to CD47 was also apparent in the affinity labeling of CD47 with ¹²⁵I-4N1K peptide (Fig. 4C). Thus, two very different methods both give evidence of a negative heterotropic effect of GTP and AlF₄ on agonist binding to CD47. These results indicate that not only does the CD47-integrin complex include a G_i -like heterotrimeric G protein, but there is a direct coupling between CD47 and the G protein.

4N1K Decreases Intraplatelet Cyclic AMP via G_i —We have shown that 4N1K activates platelets via a signaling pathway, which includes pertussis toxin-sensitive activation of SYK (10). Elevated levels of intraplatelet cyclic AMP inhibit activation, and a key feature of activation is a rapid decrease in cAMP levels. Thus, platelets maintained in prostaglandin E₁, whose receptor is coupled to G_s , are prevented from activating (19). We tested the effect of CD47 activation on cyclic AMP levels in suspended, unstirred platelets, thus preventing their aggregation and the subsequent wave of integrin-dependent outside-in signaling (19). As seen in Fig. 5, the prostaglandin E₁-treated platelets have a high level of intracellular cyclic AMP (CTL), and the control peptide 4NNGG has no effect. In contrast, 4N1K (50 μ M) treatment for 1 or 15 min dramatically lowers intraplatelet cyclic AMP levels to nearly the same extent as 5 μ M thrombin receptor peptide, a strong activator of platelets. Upon treatment of the platelets with 100 ng/ml of *pertussis* toxin for 2 h prior to stimulation, the effect of 4N1K on cyclic AMP levels is obliterated. Thus, not only does CD47 associate with G_i , but it also acts via G_i to rapidly decrease intraplatelet cyclic AMP even in the face of the elevated levels stimulated by prostaglandin E₁.

CD47 Ligation Stimulates GTP γ S Binding to Membranes—Bona fide G protein-coupled receptors are able to stimulate the exchange of GDP for GTP on the $G_{i\alpha}$ subunit of heterotrimeric G proteins. Thus, we tested the ability of the CD47 agonist

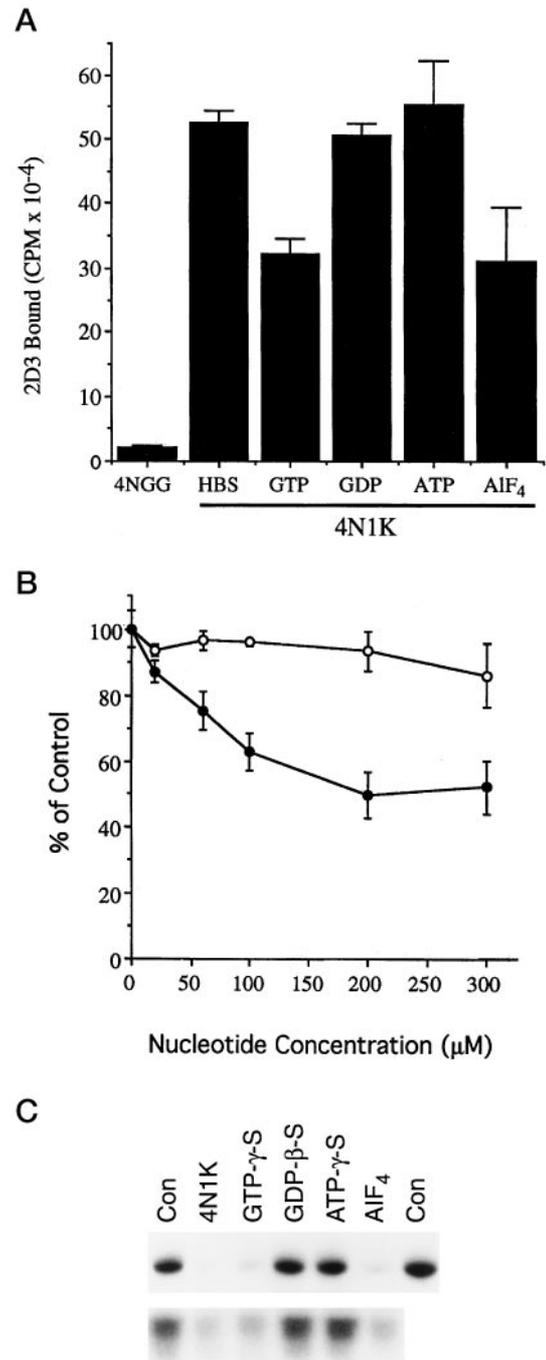


FIG. 4. Effect of nucleotides and aluminum fluoride on the binding of CD47 to 4N1K. A, a Triton X-100 (1% w/v)-soluble fraction of C32 membranes was incubated with His₆-4N1K-charged nickel-NTA beads (or His₆-4NNGG control beads) in the presence of the indicated nucleotides or AlF₄, all at 60 μ M. The beads were rapidly washed, incubated with ¹²⁵I-mAb 2D3 against CD47 for 1 h, and counted in a γ -counter. The experiment was repeated twice with similar results. B, using the same method as in A, the concentration dependence of the effect of GTP γ S and GDP β S on the binding of CD47 to the His₆-4N1K beads was determined. This experiment was repeated twice with identical results. C, a crude membrane preparation of C32 cells was affinity-labeled with ¹²⁵I-4N1K as in Fig. 1 (3, 4, 11) except that the indicated nucleotides and AlF₄ were present during the labeling reaction at 300 μ M. Con indicates no additions, and 4N1K (second lane) was present in a 50-fold molar excess during the labeling reaction to determine specificity of labeling of CD47. Autoradiograms of two separate experiments are shown.

4N1K to stimulate GTP γ S binding to membranes prepared from K562 cells, which express relatively large amounts of CD47 and $\alpha_v\beta_3$ (13). As seen in Fig. 6A, the CD47 agonist

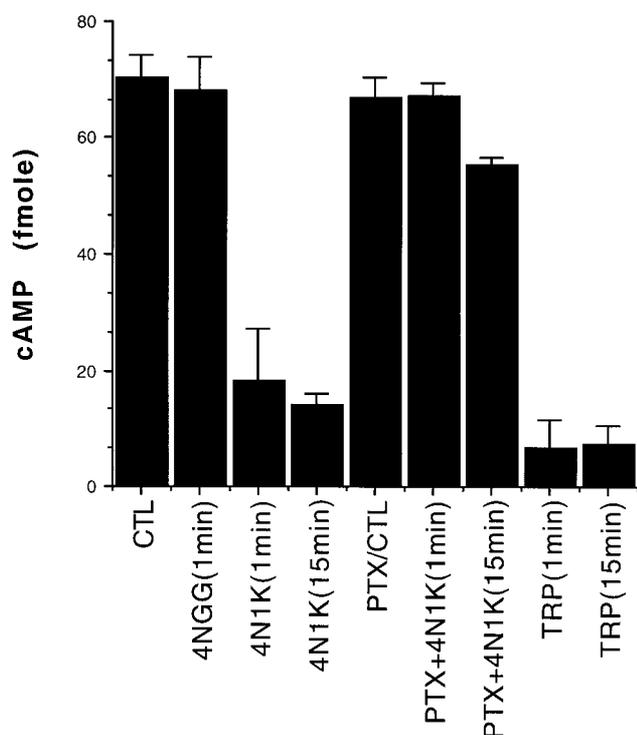


FIG. 5. **4N1K depresses intraplatelet cyclic AMP levels.** Human platelets ($2 \times 10^8/ml$) were incubated with or without pertussis toxin (PTX) at 200 ng/ml for 2 h in the presence of 100 nM prostaglandin E_1 . They were then treated in suspension without stirring (to prevent aggregation) in the presence of the indicated stimuli for 1 or 15 min, and the level of intraplatelet cyclic AMP was determined. 4NGG and 4N1K were at 50 μM , and thrombin receptor peptide (TRP) was at 5 μM . These concentrations were determined to give maximal responses.

peptide 4N1K, but not the biologically inactive control peptide 4NGG (KRFYGGMWKK), stimulated the rate of association of the radiolabeled GTP analog with membranes. The stimulation of $GTP\gamma^{35}S$ binding by the agonist peptide occurs during the early part of the time course as observed for other G protein-coupled receptors (20). G_i is quite abundant in these cells (not shown), thus accounting for the robust, $\sim 100\%$ stimulation of the extent of GTP binding by 5 min. The stimulation of $GTP\gamma^{35}S$ binding is concentration-dependent with maximal stimulation at 50 μM 4N1K, while 4NGG has no effect on binding at any concentration (Fig. 6B).

To confirm that the stimulation of GTP binding by 4N1K is in fact due to binding of the peptide to CD47 and not to a direct action on the G protein or another non-receptor-mediated effect of the peptide, we used fibroblasts isolated from CD47-deficient mice (9) as a control. As seen in Fig. 6C, 4N1K stimulates GTP binding to membranes prepared from CD47-deficient fibroblasts that had been transfected with a human CD47 expression construct but not to the untransfected CD47-deficient membranes. Lysophosphatidic acid, which binds to a G protein-coupled receptor on fibroblasts was tested as a positive control and stimulated $GTP\gamma^{35}S$ binding to about 30% the level achieved with 4N1K in both CD47-deficient and CD47-containing membranes (Fig. 6C, LPA). We also tested the effect of mastoporans, peptides reported to directly stimulate GTP binding to G proteins, and observed only a minor increase in GTP binding to K562 membranes (less than 10% of the increase seen with 4N1K, data not shown).

DISCUSSION

The coupling of CD47 to G_i protein activation is consistent with our knowledge of the biological actions of TSP and CD47. CD47 functions as a chemotaxis receptor (3) for TSP and 4N1K,

and many receptors for chemotactic ligands are 7TMS receptors that are coupled to G (usually G_i) proteins (18). CD47 on platelets is a costimulatory receptor for $\alpha_{IIb}\beta_3$ activation (10). Other platelet receptors that costimulate $\alpha_{IIb}\beta_3$ (e.g. thrombin, ADP, epinephrine, and thromboxane receptors) are 7TMS receptors coupled to heterotrimeric G_i proteins (19). Activation of all of these costimulatory receptors in platelets leads to a precipitous drop in intraplatelet cAMP levels, an event essential for further activation and aggregation (19). As shown in Fig. 5, activation of CD47 on platelets results in an immediate and profound decrease in platelet cAMP.

Other signaling events thought to emanate from CD47 include Ca^{2+} fluxes (6) and activation of protein kinase C and phosphatidylinositol 3-kinase (4). All of these events are regulated by 7TMS receptors coupled to heterotrimeric G proteins (21). Interestingly, all of these activities have also been implicated in integrin regulation in a variety of systems (22). Our recent data show that CD47 can associate with and/or modulate integrins of the β_1 (11) and β_2 families (23, 24),² as well as β_3 (3, 4, 10). In all of these biological systems, the effects of the CD47 agonists 4N1K and TSP-1 on cell spreading, chemotaxis, and adhesion are blocked by pertussis toxin treatment of the cells in question (4, 10).^{2,3} Thus, it appears that different integrin $\alpha\beta$ heterodimers associating with CD47 can all couple to G_i protein-dependent pathways. This combinatorial diversity of the integrins that can associate with and be regulated by CD47 may underlie some of the long noted, yet poorly explained, variety of functions attributed to TSP proteins (1, 2). The agonist peptide sequence RFYVVMWK is extremely well conserved in all five TSP isoforms; thus, CD47 is probably a receptor for all TSP family members.

Aside from the physical association of integrins, CD47, and G_i proteins, other factors support the concept of a functional association in which CD47 communicates directly with G_i . First, treatment of platelets (Fig. 5) with 4N1K initiates a precipitous drop in intraplatelet cyclic AMP levels. This signaling event is common to all of the agonists that activate platelets through G protein-coupled receptors (19). We have also observed a similar fall in intracellular cyclic AMP in other cell types that respond to TSP-1 stimulation with integrin activation.² Second, the agonist peptide 4N1K rapidly stimulates $GTP\gamma^{35}S$ binding to membranes (Fig. 6). These membranes contain few means of generating a signal intermediate between CD47 ligation and G_i activation, since triphosphate kinase substrates and energy-requiring reactions are eliminated. Last, a direct link between CD47 and G_i is indicated by the negative heterotropic effect of G protein activators ($GTP\gamma S$ and AlF_4) on the binding of 4N1K to CD47.

Based on the data presented here, we propose a model for CD47 action in which the integrin-CD47 heterotrimer functions as an *ad hoc* serpentine or 7TMS receptor, each of the integrin subunits contributing one transmembrane segment and CD47 contributing its five. This *heterotrimeric* receptor would then activate heterotrimeric G proteins in much the same way as classical 7TMS receptors. This hypothesis garners support from a number of additional observations as follows. (i) The stability of the integrin-CD47 complex is consistent with the formation of a seven-helix bundle within the core of the membrane. The integrin-CD47 complex survives solubilization with several different detergents (including radioimmune precipitation buffer under some conditions) and the extensive washing associated with affinity chromatography. SDS gels of material eluted from the 4N1K columns reveal the integrin

² W. A. Frazier, A.-G. Gao, J. Dimitry, J. Chung, E. J. Brown, F. P. Lindberg, and M. E. Linder, unpublished data.

³ X. Q. Wang and W. A. Frazier, manuscript in preparation.

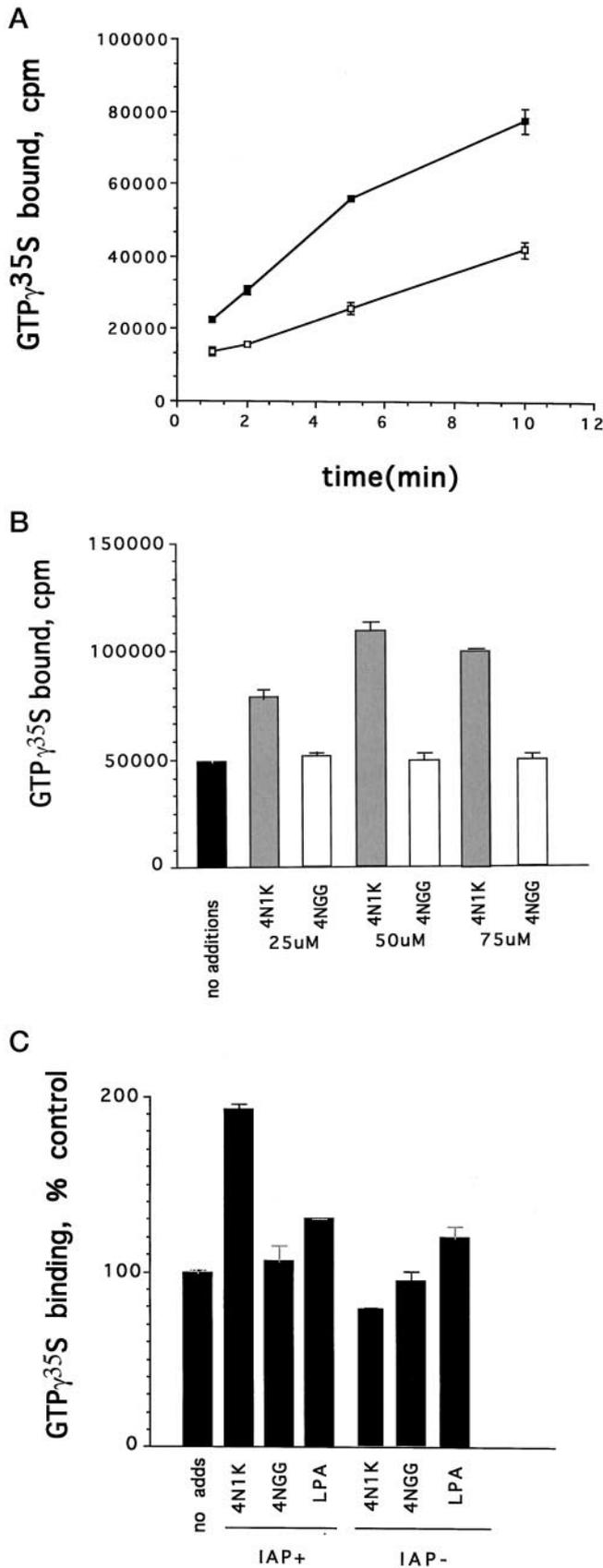


FIG. 6. 4N1K stimulates $GTP\gamma^{35}S$ binding to membranes. *A*, membranes of $\alpha_v\beta_3$ -K562 cells were incubated with the labeled GTP analog (~ 5 nM) and either 4N1K or 4NGG ($50 \mu M$) at $30^\circ C$ as described under "Experimental Procedures." At the indicated times, samples were removed in triplicate, rapidly added to 3 ml of ice-cold buffer, and filtered through nitrocellulose filters, which were washed with another 3 ml of ice-cold buffer. The filters were dried and counted. Background

chains and CD47 as the major protein-stained bands.⁴ (ii) Methods that allow detection of CD47-G protein complexes from C32 and platelet membranes detect little or no CD47-G protein complex when applied to red blood cell membranes.⁵ Red cells contain both CD47 and G_i proteins but no integrins (25), suggesting that the integrin may be necessary to stabilize the CD47-G protein complex. However, there may be integrin-independent actions of CD47 in other cells such as T cells (26). (iii) The bundle of seven transmembrane segments need not be covalently connected for even classical G protein-coupled receptors to bind ligand and activate G proteins. Proteolysis of the β -adrenergic receptor results in disconnected transmembrane segments fully capable of signaling (27). In addition, a functional β_2 -adrenergic receptor was assembled by expression of two separate proteins, one containing transmembrane segments 1–5 and the other containing segments 6 and 7 (28). (iv) Structural features conserved across the large 7TMS receptor superfamily have some homologs in integrin-CD47 heterotrimers. These include the DRY sequence, which occurs at the membrane-cytoplasmic boundary of the third transmembrane segment of many 7TMS receptors, the WXXXL sequence in transmembrane segment IV, and positively charged residue clusters in cytoplasmic domains involved in G protein coupling (29). Further experiments employing mutagenesis strategies are under way to map the sites of G protein coupling in CD47 and thus test this hypothesis. (v) The CD47 agonist peptide 4N1K is similar in sequence to peptide ligands for other G protein-coupled receptors. For example, the neurokinin I receptor, which can couple to G_i , binds short peptide ligands (30). Among them is physalaemin with the sequence -KFYGLM, which is similar to the sequence -RFYVVM of 4N1K/TSPs. Taken together, these considerations suggest that a functional heterotrimeric 7TMS unit could be assembled from the α - and β -chains of an integrin associated with the five TM segments of CD47. This model is currently being tested.

In summary, we report that the CD47-integrin complex includes one or more heterotrimeric G_i proteins. We provide functional evidence that there is a direct interaction between CD47 and the G protein as evidenced by the following: (i) a negative heterotropic effect of GTP and AlF_4 on the binding of 4N1K by CD47; (ii) the fact that 4N1K treatment of cells causes a rapid, pertussis toxin-sensitive drop in intracellular cyclic AMP; and finally (iii) 4N1K specifically stimulates the binding of a radiolabeled GTP analog to membranes in a CD47-dependent fashion. Given the ubiquitous distribution of integrins, CD47, and TSP family members, these results provide a far reaching paradigm for many of the biological actions of TSP proteins and suggest a novel route for G protein activation leading to the modulation of integrin function in many systems.

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⁴ J. Chung and W. A. Frazier, unpublished data.

⁵ W. A. Frazier, A.-G. Gao, J. Dimitry, J. Chung, E. J. Brown, F. P. Lindberg, and M. E. Linder, unpublished results.

binding to the filters (no membranes) was less than 1000 cpm. *B*, concentration dependence of the stimulation of GTP binding by 4N1K. Assays were performed with $\alpha_v\beta_3$ -K562 membranes as in *A* with an incubation time of 10 min at the indicated concentrations of 4N1K or 4NGG. *C*, the stimulation of GTP binding by 4N1K requires CD47. Fibroblasts isolated from CD47-deficient (CD47^{-/-}) mice (Ref. 9; F. Lindberg, unpublished results) were transfected with a CD47 expression construct or the empty vector as described by Lindberg *et al.* (31). CD47 expression was confirmed by fluorescence-activated cell sorting analysis. Membranes isolated from the CD47-transfected and control cells as described under "Experimental Procedures" were assayed for $GTP\gamma^{35}S$ binding as above using $50 \mu M$ 4N1K or 4NGG. Lysophosphatidic acid (LPA; $10 \mu M$) was used as a positive control.

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