Integrin-associated protein (CD47) and its ligands

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Integrin-associated protein (IAP or CD47) is a receptor for thrombospondin family members, a ligand for the transmembrane signaling protein SIRPα and a component of a supramolecular complex containing specific integrins, heterotrimeric G proteins and cholesterol. Peptides containing a VVM motif in the C-terminal domain of thrombospondins are agonists for CD47, initiating heterotrimeric Gi protein signaling that augments the functions of integrins of the β1, β2 and β3 families, thus modulating a range of cell activities including platelet activation, cell motility and adhesion, and leukocyte adhesion, migration and phagocytosis.

The regulated and specific interaction of several individual proteins to form a complex with new functions is a well-established principle of signal transduction. The binding of a ligand to its receptor results in the assembly of complexes of cytoplasmic molecules required to generate the cascades that transfer information from the extracellular milieu to the nucleus. It is now apparent that such multimolecular complexes also exist within the plane of the plasma membrane. Integrins in particular form complexes with growth-factor receptors, tetraspanin molecules and some GPI-linked proteins[1–4]. Perhaps the best-studied integrin-containing plasma membrane supramolecular complex is that of the integrin αvβ3 with the pentaspanin integrin-associated protein (IAP or CD47). This complex has unique functional capabilities due at least in part to its unique signal-transduction properties. It is now clear that CD47 interacts with other integrins in addition to αvβ3 and also has its own extracellular ligands. In this review, we discuss the biology of CD47, focusing especially on the molecular mechanisms by which it can modulate integrin functions.

The discovery of CD47 as an integrin-associated molecule

Integrin-associated protein was discovered originally as a plasma membrane molecule that copurified with the integrin αvβ3 from leukocytes and placenta[5]. Monoclonal antibodies (mAbs) against the placental protein could block the signaling function of αvβ3 on polymorphonuclear leukocytes (PMN), and ligation of CD47 with activating antibodies induced signaling identical to that resulting from ligation of αvβ3. From the evidence of coprecipitation, similar function and cross-inhibition arose the hypothesis that αvβ3 and CD47 could function as a signaling complex on PMN[5,6]. Further studies demonstrated that there was a relatively stable interaction between CD47 and αIIbβ3 or αvβ3 on platelets[7], αvβ3 on melanoma cells[8] and ovarian carcinoma cells[9,10], and α2β1 on smooth muscle cells[11] and platelets[12]. CD47 is expressed ubiquitously, and, after its cDNA was cloned and expressed, antibodies to CD47 were shown to recognize integrin-associated protein, demonstrating that the two molecules were the same[13]. Molecular cloning also demonstrated that CD47 was identical to a cancer antigen, OV-3, markedly upregulated on ovarian carcinoma cells compared with normal ovarian cells[6]. Because the function of this molecule has been studied best in relation to integrin signaling, the name IAP is frequently used, as is the less informative CD47. Now that it has been shown to interact with molecules in addition to integrins, the unbiased name CD47 is perhaps more appropriate.

The cluster determinant CD47 was first described by investigators seeking the Rh polypeptide[14]. Several monoclonal antibodies that recognized normal, but not rare Rh-null, erythrocytes were found to bind to CD47, a highly glycosylated ~50-kDa plasma membrane protein. However, it was quickly realized that CD47 was not the product of the Rh gene, but one of several molecules (RhD, RhCE, CD47, LW, Duffy and glycophorin B) that were expressed poorly on erythrocytes from individuals with defective genes for the Rh50 polypeptide. CD47 is a broadly expressed antigen, present on many different cell types in all tissues, and Rh-null individuals have normal CD47 levels on cells other than erythrocytes. The basis for the failure of CD47 expression on Rh-null erythrocytes is not understood. Recently, it has been shown that CD47-deficient erythrocytes are cleared rapidly from the bloodstream of normal, but not CD47-deficient, mice[15]; this might explain the hemolytic anemia of Rh-null individuals.

CD47 structure

CD47 is an unusual member of the immunoglobulin (Ig) superfamily of membrane proteins, with a single IgV-like domain at its N-terminus, a highly hydrophobic stretch with five membrane-spanning segments [the multiply membrane-spanning (MMS) domain] and an alternatively spliced cytoplasmic C-terminus ranging in length from 3–36 amino acids (Fig. 1)[6]. Mouse, rat, bovine and human CD47 molecules have been cloned and show about 70% overall amino acid identity. Genomic Southern blots suggest that homologous genes are present in other vertebrate species too, but no homologs have been

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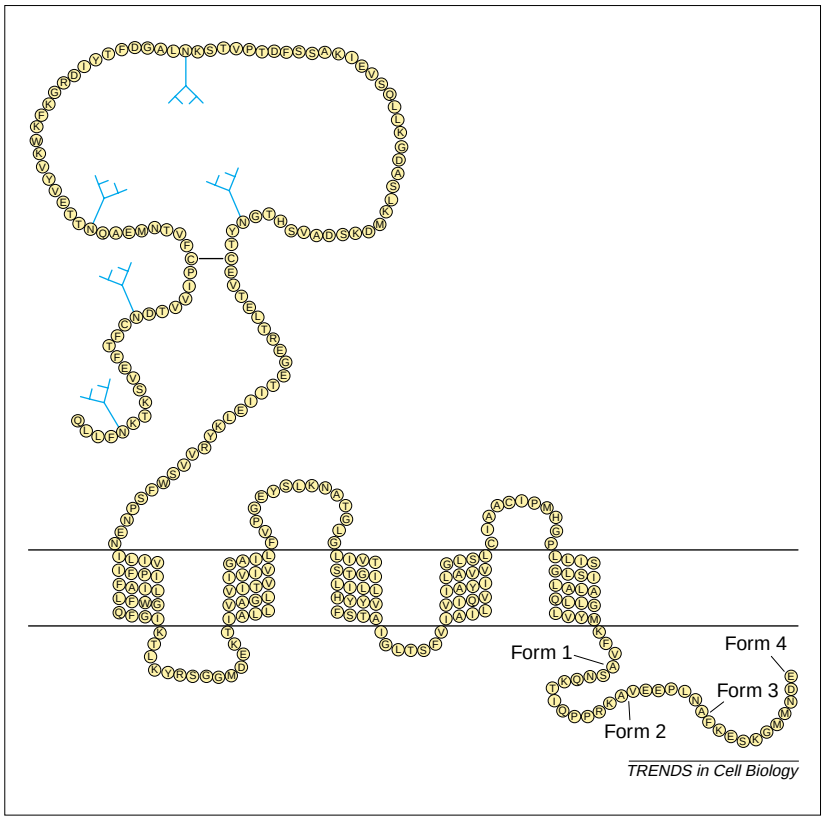

Fig. 1. Structure of integrin-associated protein (IAP/CD47). The heavily glycosylated extracellular immunoglobulin variable (IgV) domain is followed by five probable transmembrane segments terminating in a cytoplasmic tail that is alternatively spliced, giving rise to four isoforms, the longest of which, form 4, is shown in its entirety.

found in invertebrates. All pox viruses encode an open reading frame with homology to CD47, suggesting that this gene might play a role in the pathogenesis of these viruses. However, the gene has been deleted in vaccinia, with no apparent effect on virulence[16].

The calculated molecular weight for human CD47 is 31871–35213, depending on the length of the C-terminal cytoplasmic tail, but the Ig domain is heavily glycosylated, leading to its broad migration at 45–55 kDa on SDS–PAGE. Some murine CD47 cDNAs encode a sequence of 20 amino acids in the extracellular portion of the molecule that has not been found in other species. Whether this represents a true protein variant and whether it might have different functions compared with the shorter 'standard' form is not known. The Ig domain is required for CD47 interaction with its associated integrins αvβ3 and α2β1 and its ligands thrombospondin (TSP) and signal-regulatory protein alpha (SIRPα). Each of the four alternatively spliced cytoplasmic tails (Fig. 1) has been found *in vivo*[17]; the second-shortest, the so-called form 2, is by far predominant. The second most abundant isoform, form 4, has the longest cytoplasmic extension and is found primarily in neurons, intestine and testis. None of the CD47 cytoplasmic extensions has any known motif for enzymatic activity or protein interaction.

Recently two cytoplasmic proteins have been described, termed PLICs (for: *p*roteins *l*inking *I*AP to *c*ytoskeleton), that bind to both the form 2 and form 4 cytoplasmic tails[18]. Overexpression of both PLIC-1 and -2 increases cell spreading and alters the distribution of intermediate filaments. PLIC-1 and PLIC-2 are closely related to each other in primary sequence, and have ubiquitin-like N-termini, and UBA domains[19] at their C-termini. They are members of a highly conserved family of proteins found throughout evolution from yeast to man and might be involved in linking the ubiquitin conjugation pathway to the proteasome[20]. The role of PLICs in CD47 signaling and function is as yet unclear, although, given the effects of CD47 on cell spreading and motility[8,11], a role for PLICs in cytoskeletal regulation is appealing.

CD47 ligands

When two plasma membrane molecules interact, cell–cell adhesion or communication generally results. The interaction between CD47 and those integrins with which it associates seems, however, to be quite distinct. These molecules interact within a single plasma membrane to make a complex possessing signaling properties absent from the individual components outside the complex. This sort of *cis* interaction might be more common for integrins than appreciated previously because urokinase plasminogen activator receptor (uPAR), caveolin and various tetraspanin molecules all can interact in *cis* with various integrins in the same plasma membrane[1–4]. CD47 does not interact with all integrins. To date, only the broadly expressed RGD receptor αvβ3[7,8,10], the platelet fibrinogen receptor αIIbβ3[7] and the collagen receptor α2β1[11] have been coprecipitated or copurified with CD47. The structural requirements for coprecipitation and functional cooperation have been examined carefully only for CD47 association with αvβ3. In this case, the Ig domain of CD47 is required for both functional and physical interaction. Cells expressing the Ig domain attached to the plasma membrane with a glycan phosphoinositol anchor or a CD7 single-pass transmembrane segment did not coprecipitate with αvβ3 but could restore αvβ3 binding function[21]. This suggests that coprecipitation requires a higher-affinity interaction between the two molecules than functional association. While the Ig domain is required for association, the MMS domain apparently stabilizes association significantly. The mechanism of the MMS effect is likely to involve its ability to bind to cholesterol because cholesterol is required for stable, immunoprecipitable association of CD47 with αvβ3[10].

Binding to SIRPα

Recently the plasma membrane protein SIRPα has been shown to be a CD47 ligand[22]. Unlike integrin–CD47 interactions, which apparently occur only within a single plasma membrane, CD47–SIRPα interactions can mediate cell–cell adhesion[23]. SIRPα is a member of the Ig superfamily, with three Ig-like domains in its

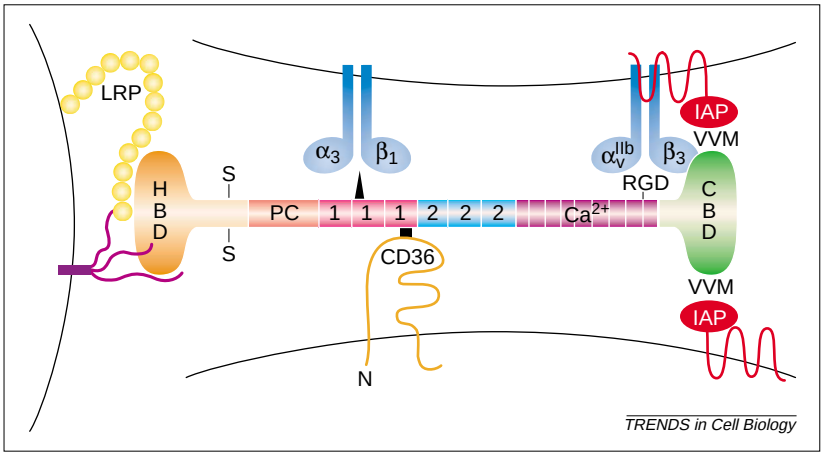

Fig. 2. The structure of the thrombospondin 1 (TSP-1) subunit and its receptors. The TSP-1 peptide chain consists of (N- to C-terminal): 1) a heparin-binding domain (HBD) that binds to cell-surface proteoglycans (purple) and lipoprotein-receptor-related protein (LRP, yellow), 2) a domain related to the N-terminal propeptide of collagens (PC) that is the collagen-binding domain of TSP-1, 3) three type 1 repeats that contain binding sites for α3β1 integrin and CD36, 4) three type 2 or epidermal growth factor (EGF)-like repeats thought to be responsible for the interaction of TSP-1 with soluble and matrix proteins, 5) a highly repetitive Ca^{2+}-binding region that contains the single RGD sequence of TSP-1 that binds to integrins αIIbβ3 and αvβ3, and 6) the C-terminal cell-binding domain (CBD), which contains the two VVM sequences that bind to integrin-associated protein (IAP/CD47). Note that the proximity of the RGD and VVM motifs might allow for simultaneous engagement of the β3 integrin and CD47.

extracellular component. Its cytoplasmic domain contains several tyrosines that, when phosphorylated, can create an immunoreceptor tyrosine inhibitory motif (ITIM). ITIMs are sites for binding of the dual Src-homology 2 (SH2)-domain-containing tyrosine phosphatases, SHP-1 and SHP-2, and the inositol phosphatase SHIP. SIRPα seems to act primarily as a site for the recruitment of tyrosine phosphatase activity to the membrane, leading to inhibition of signaling from growth-factor receptors[24] and perhaps other receptors that promote tyrosine kinase activity. CD47-deficient circulating cells are cleared rapidly by splenic macrophages because this inhibitory signal is lacking, and binding of the blood cells to the macrophages is sufficient to trigger a phagocytic signal[15]. It has been shown recently that SIRPα–CD47 interactions in *trans* play a role in macrophage fusion, resulting in osteoclasts and giant cells[25].

Binding to thrombospondins

The thrombospondins comprise a family of five genes encoding proteins designated TSP-1 through TSP-5[26]. Platelet thrombospondin, or TSP-1 as it is now known, is the prototypic member of this family. The complete cDNA sequence of TSP-1 provided the basis for detailed studies of the structure–function relationships of this complex and interesting molecule[26] (Fig. 2). TSP-1 and -2 are trimeric and have identical domain structures, whereas TSPs 3–5 are pentamers and are missing the procollagen-like and type 1 repeat regions found in TSP-1 and -2. The exons encoding these regions are absent from the genes encoding TSPs 3–5[26,27]. TSPs -1 and -2 have a complex pattern of widespread expression during embryogenesis, whereas TSPs 3–5 appear to be expressed in more localized fashion at specific stages of development[27].

To unravel the many interactions of TSPs with receptors on cells, it has been necessary to focus on one binding site at a time. This has been done by generation of proteolytic fragments of TSP-1 and later by expression of recombinant domains. In addition, a battery of monoclonal antibodies has helped to identify and locate cell interaction sites within the protein. A major cell attachment site was identified in the extreme C-terminal domain of TSP-1[28]. Using synthetic peptides, the cell-binding activity was localized to two sequences both containing the unlikely adhesion motif VVM. One of these peptides, 4N1 or RFYVVMWK, is highly conserved in all species and isoforms of TSP[29]. Affinity labeling identified a 50-KDa membrane glycoprotein as a receptor candidate for the peptide on many cell types that proved to be CD47[30]. Since the CD47-binding sequence occurs in all TSP isoforms, we believe that CD47 is a receptor for all TSP family members.

How does CD47 affect ligand binding by integrins?

Cells expressing αvβ3 without CD47 adhere to surfaces coated with vitronectin (Vn) but do not bind to Vn presented on latex beads[21]. Moreover, CD47-deficient cells plated onto Vn spread less well than when CD47 is present, and they migrate poorly on either α2β1 or αvβ3 substrates[31]. CD47-expressing cells spread much more rapidly on low-density Vn when CD47 is ligated by TSP, the TSP C-terminal domain or 4N1K peptide[8]. The molecular basis for these effects of CD47 on αvβ3 ligand binding are not entirely clear, although they likely involve activation of Gαi-containing heterotrimeric GTPases because of an inhibitory effect of pertussis toxin on signaling[9,10,31]. The affinity of αvβ3 for RGD, and RGD-induced conformational change in the integrin appear unaffected by presence or absence of CD47, and αvβ3 localization to cholesterol-rich membrane rafts does not require CD47[10]. It is possible that CD47 affects the ability of integrins to cluster upon ligand binding, but CD47 is not present at focal contacts – adhesion sites where clustered integrins attach to the actin cytoskeleton. However, recent data demonstrate the presence of CD47, αvβ3 and Gi in early 'adhesion complexes' at the edges of spreading melanoma cells and human vascular endothelial cells (HUVECs; J. Chung, T. Mariani and W. Frazier, unpublished).

CD47 on leukocytes

CD47 is present on all leukocytes, and its first functional description was as part of a complex with αvβ3 that led to neutrophil activation by extracellular matrix. CD47-deficient neutrophils do not activate normally in response to extracellular matrix ligands for αvβ3[32]. On monocytes, the αvβ3–CD47 complex binds to soluble CD23 and signals cytokine synthesis in response to binding[33]. By contrast, several recent publications have suggested that ligation of CD47 inhibits macrophage and dendritic cell cytokine synthesis[34,35]. Inhibition can be induced by ligation of

CD47 with TSP or antibody without apparent need for any integrin ligand, raising the possibility that it is an integrin-independent function for CD47. This and the discovery that SIRPα–CD47 interactions can mediate cell–cell adhesion have increased interest in the possibility that CD47 might sometimes act in ways independent of its association with integrins. However, it has recently been shown that ligation of αvβ3 can induce production of transforming growth factor (TGF-β) by macrophages[36]. Because TGF-β is a potent inhibitor of macrophage synthesis of proinflammatory cytokines, the possibility remains that CD47 inhibition of macrophage proinflammatory function requires the αvβ3–CD47 complex.

There is evidence that CD47 has integrin-independent functions on lymphocytes. Ligation of CD47 on B cell lymphomas leads to apoptosis, and this apparently is distinct from ligation of αvβ3 on these cells[37]. Ligation of T cell CD47 can synergize with the antigen receptor to activate synthesis of interleukin 2 (IL-2), an effect not reproduced by ligation of αvβ3 or α2β1[38–40]. Because the CD47 ligand SIRPα is highly expressed on macrophages and dendritic cells[41], it is possible that CD47 has a physiologic role in T cell stimulation. There is a decrease (~50%) in the number of T cells in the spleens of CD47$^{-/-}$ mice[21], but the basis for this abnormality is not known.

CD47 signal transduction

CD47 is a TSP receptor on platelets that activates the fibrinogen-binding integrin αIIbβ3. The CD47 agonist peptide 4N1K, the recombinant C-terminal domain of TSP1 containing the VVM sequences and intact TSP1 all stimulate platelet spreading on fibrinogen. This spreading is mediated by αIIbβ3 and is prevented by a function-blocking mAb against CD47[7]. 4N1K is a potent activator of platelets[7,42] and leads to activation of αIIbβ3 integrin as assessed by the binding of the ligand-mimetic mAb PAC-1[7]. In addition, CD47 stimulation synergizes with soluble collagen, enhancing this α2β1-dependent activation of platelets[12]. As with other platelet co-stimulators such as thrombin, epinephrine and ADP, the spreading response to CD47 ligands is blocked by inhibitors of protein kinase C (PKC) and cyclooxygenase, by cytoskeletal poisons and by pertussis toxin. In platelets allowed to spread on fibrinogen, 4N1K rapidly stimulates phosphorylation of focal-adhesion kinase (FAK) and the Src kinase Lyn. The tyrosine kinase Syk is also phosphorylated very rapidly upon stimulation with 4N1K, even in suspended platelets prevented from aggregating. The only inhibitor that significantly reduced tyrosine phosphorylation of Syk in these experiments was pertussis toxin, suggesting a rather direct link from Gi activation to activation of Syk kinase[7]. Recent data suggest that this might be due to a Gi–Src interaction[43], leading to Src-dependent activation of Syk.

CD47 ligation stimulates α2β1-mediated chemotaxis of SMCs

There is a body of data suggesting that TSP-1 is an important regulator of smooth muscle cell (SMC) migration[44] and proliferation[45,46] *in vitro*, and recent studies indicate a role for TSP-1 in the neointimal response of injured arteries[47]. Thus it was of interest to determine whether TSP, acting through CD47, could direct the migration of SMCs by regulating the action of SMC integrins. Both human and rat aortic SMCs attach and migrate on both gelatin and collagen matrices using α2β1. In a Boyden chamber chemotaxis assay, migration is weakly stimulated by either 4N1K peptide or soluble native type I collagen. However, TSP1 or 4N1K dramatically synergize with soluble collagen to provoke an aggressive chemotactic response of the SMCs[11]. This increased migration is blocked by mAbs recognizing either α2β1 integrin or CD47. In SMCs from CD47-deficient mice, 4N1K and TSP1 are not chemoattractants, and 4N1K does not stimulate migration towards soluble collagen[31].

Migration towards 4N1K and collagen is completely blocked by treatment of the SMCs with pertussis toxin to inactivate Gi[31]. As expected, because the classical, obvious target of Gi is adenylate cyclase, 4N1K or TSP1 treatment of SMCs causes an immediate and dramatic fall in intracellular cAMP levels, which is prevented by pertussis toxin. Forskolin, which directly activates adenylate cyclase, and 8-Br cAMP both inhibit chemotaxis to 4N1K with or without soluble collagen, indicating that diminished cAMP levels are necessary for chemotaxis. This hypothesis is consistent with the well-established inhibition of integrin activation in both platelets[48] and leukocytes[49] by elevated cAMP levels. To our surprise, activation of ERK1 and ERK2 also is strongly inhibited by 4N1K, and pertussis toxin treatment of SMC stimulates ERK activity. Inhibition of ERKs appears to be essential for chemotaxis as well because the MEK inhibitor PD098059 stimulates the chemotactic response to 4N1K. In addition, reduction of ERK levels with antisense oligonucleotides or transfection of SMCs with MAP kinase phosphatase 1 both enhance the magnitude of the CD47-dependent chemotactic response. Thus, it appears that both low cAMP levels and decreased ERK activity are necessary for a robust chemotactic response of SMCs mediated by α2β1[31]. CD47-dependent modulation of ERK and α2β1 integrin in SMCs might well be the first physiological example of ERK-mediated integrin regulation.

CD47 signals via heterotrimeric Gi

In view of the effect of pertussis toxin on CD47 stimulation of cell spreading, platelet activation and chemotaxis, we investigated the potential functional and physical association of CD47 with heterotrimeric G proteins of the Gi family. Antibodies to the G protein alpha subunit could immunoprecipitate affinity-labeled CD47, and mAbs against CD47 could immunoprecipitate alpha and beta G protein subunits. Treatment of cells with pertussis toxin eliminated the co-immunoprecipitation of CD47 and Gi[9]. The evidence

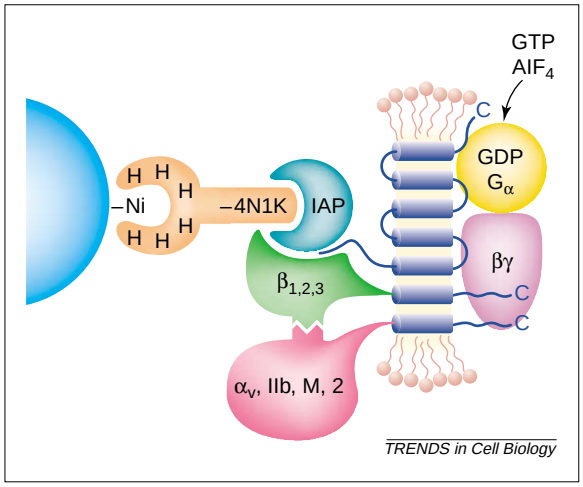

Fig. 3. The integrin–CD47 signaling complex. The cartoon depicts the TSP-1 peptide 4N1K in His-tagged form bound to a Ni-NTA bead. The 4N1K has bound to CD47 with one of its associated integrins, thus immobilizing this seven-transmembrane segment structure on the beads along with associated lipid molecules, including cholesterol and the heterotrimeric G protein. Addition of either GTP or AlF_4 elutes the G-protein from the beads[9].

supporting a model of CD47–Gi activation is summarized in Box 1, along with a depiction of the detergent-solubilized integrin–CD47 complex, heterotrimeric G protein and associated lipids bound to 4N1K peptide immobilized on a Ni-NTA column (Fig. 3). This figure also illustrates how the five transmembrane segments of CD47 along with the two TM segments of its partner integrin might form an *ad hoc* seven-transmembrane-spanning (7TMS) complex that could function in G protein activation. An attractive feature of the 7TMS model is that different integrin partners and/or CD47 isoforms that differ in their cytoplasmic tails might influence the ability of the complex to activate specific heterotrimeric G proteins[9].

Another component of the CD47–integrin–G-protein signaling complex is cholesterol. Treatment of the αvβ3–CD47–Gi complex with β-methyl-cyclodextrin to remove cholesterol causes complete disruption of the protein complex. Treatment of intact cells with cyclodextrin blocks coimmunoprecipitation of CD47 and Gi with αvβ3, and restoration of cholesterol to the depleted cells with preformed cholesterol–cyclodextrin complexes reconstitutes the integrin–CD47–Gi complex. Cholesterol depletion does not affect the ability of cells to spread on high-density vitronectin coatings but completely abolishes the ability of CD47 stimulation to accelerate spreading on sparse vitronectin coatings. Thus, the basic spreading machinery of the cell is not dependent on cholesterol, yet cholesterol appears to have an essential role in maintaining the functional CD47–integrin–G-protein complex[10]. That CD47 signaling depends on cholesterol is further supported by the finding that cyclodextrin treatment of cells prevents the CD47-dependent reduction in intracellular cAMP levels upon treatment with 4N1K.

In many cell types, cholesterol and some heterotrimeric G proteins are preferentially localized to low-density membrane domains termed rafts or detergent-insoluble glycolipid-enriched fractions/domains (DIGS)[50]. Using sucrose density-gradient flotation, we found αvβ3 integrin, CD47 and Gi protein subunits all to be present in the cholesterol-rich, light membrane fraction of the cells. Using cell lines devoid of each component of this complex, we found that each protein can localize to DIGS independently, where it appears that the locally high concentration of cholesterol facilitates complex assembly. The MMS domain of CD47 is important for interaction with Gi and perhaps also with the integrin[10]. However, much work needs to be done to clarify the molecular interactions among protein components of this CD47 signaling complex and to define the precise role of cholesterol and perhaps other membrane lipids in its structure and function.

Box 1. Evidence supporting a direct coupling of CD47/IAP to Gi

- CD47/integrin-associated protein (IAP), integrins and Giαβγ coimmunoprecipitate, and pertussis toxin treatment or cholesterol depletion disrupts the complex[a–d]
- CD47/IAP, integrin and Giαβγ copurify on a 4N1K affinity matrix, and GTP and AlF_4 elute Gi from the matrix[c]
- GTP (not GDP or ATP) and AlF_4 decrease 4N1K affinity labeling of CD47/IAP[c]
- CD47/IAP ligands rapidly decrease cellular cyclic AMP levels[c–e]
- In membranes, 4N1K and rCBD stimulate the binding of GTP – but only in membranes containing CD47/IAP[c]
- In three cell types, CD47/IAP, integrin and Gi colocalize to filopodia/lamellipodia of spreading and migrating cells[f]

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Concluding remarks

CD47 can associate with and modulate the activity of several families of integrins (β3, β2 and β1), and most, if not all, of these actions depend on activation of heterotrimeric G proteins. The details of the mechanism of G protein activation remain to be worked out. It is attractive to speculate that the integrin–CD47 complex acts in a way similar to a classical seven-spanning receptor, thus allowing for

the possibility that complexes of different integrins and CD47 isoforms signal through different G proteins or lead to different downstream consequences. It is clear that CD47 binds to SIRPα, leading to inhibitory signaling, but it is not known whether this interaction leads to CD47 signaling as well, perhaps in macrophage fusion[25]. Studies of CD47$^{-/-}$ mice and manipulation of CD47 expression *in vitro* have confirmed suspected roles of CD47 in leukocyte transmigration crucial for host defense[15]. Given the widespread potential for CD47 modulation of integrin function and the ubiquitous and complex expression of TSP isoforms, it is likely that new roles for CD47 will be revealed in many developmental, immunological and pathological processes.

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