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Ectodomain interactions of leukocyte integrins and pro-inflammatory GPI-linked membrane proteins

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Abstract

Although glycosylphosphatidyl–inositol (GPI) linked membrane proteins do not possess transmembrane or cytosolic sequences they elicit transmembrane signals. Using microscopic fluorescence imaging and resonance energy transfer (RET) techniques we have shown that certain pro-inflammatory GPI-linked membrane proteins can interact with leukocyte β_2 integrins (complement receptor type 3 (CR3) and 4 (CR4) and the leukocyte function-associated antigen-1 (LFA-1)). For example, physical associations between CR3 and FcγRIIIB, CR3 and urokinase receptors, and CR3 and CD14 (lipopolysaccharide receptor) have been found. Although FcγRIIIB appears to be constitutively associated with CR3, urokinase receptors and CD14 associations with CR3 are influenced by their ligation status and cell function (e.g. adherence and locomotion). CR3-to-urokinase receptor interactions have been confirmed by immunoprecipitation techniques. Immunoprecipitation of CR3 from Brij-58 lysates after biotinylation of neutrophil membranes revealed proteins of $M_r = 40\,000$, 50 000, 74 000 and 120 000, in addition to bands corresponding to the integrin α and β chains. Cell functions such as transmembrane signaling and superoxide release/priming have been linked to these interactions. Importantly, reagents that affect the lectin-like site of CR3, such as *N*-acetyl-D-glucosamine, α-methyl-D-mannoside and β-glucan alter these interactions and, in parallel, leukocyte functions. Thus, the interactions of GPI-linked proteins and integrins can be highly dynamic events linked to cell activities. Our studies suggest that it may be possible to develop new drugs directed at the lectin-like site of β_2 integrins that block GPI-linked protein-to-integrin coupling thereby controlling inflammatory cell processes including cell adherence, locomotion and activation. Such drugs may be useful in clinical conditions such as ischemia-reperfusion injury, sepsis, arthritis and others. © 1997 Elsevier Science B.V.

Keywords: Ectodomain; Leukocyte; Integrins; Pro-inflammatory

1. Introduction

Conventional biological membrane signal transduction is generally envisioned as a ligand–recep-

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Table 1
Lateral interactions of public transducers in membrane signaling

Public transducer	Private receptor	Expression	Reference
CR3	FcγRIIIB	GPI	[14–17]
		Soluble	[27]
	uPAR	GPI	[18–21,56,61]
	CD14 (LPS/LBP)	GPI	[22]
	FcγRII	Transmembrane	[73,76]
	FcεRII	Soluble	[78]
gp130	IL-6	Transmembrane	[2–4]
		Soluble	[3]
	IL-11	Transmembrane	[2,4]
	Oncostatin M	Transmembrane	[2,4]
	Leukemia inhibitory factor	Transmembrane	[2,4,80]
	Ciliary neurotrophic factor	GPI	[2,4,80,83]

tor binding event followed by a transmembrane signal that triggers chemical changes at the internal face of a membrane. For example, insulin-to-insulin receptor binding leads to phosphorylation reactions and catecholamine binding to adrenergic receptors activates intracellular G proteins [1]. This conventional paradigm, however, has recently been challenged by a variety of experimental findings. A common theme among these newer signaling schemes is that one plasma membrane receptor with a single or 'private' specificity recognizes a ligand whereas a second receptor or protein acts as a 'public' transducer of multiple signals (Table 1). Genetic complementation studies have suggested that the transmembrane interleukin-6 (IL-6) receptor, and other receptors as well, mediate ligand binding while another membrane protein, gp130, mediates signal transduction [2–4]. The conventional paradigm has also been challenged by studies showing that glycosylphosphatidyl–inositol (GPI)-linked membrane receptors, which lack transmembrane and cytoplasmic sequences, elicit transmembrane signals and physiological functions [5–11]. The public transducers for several GPI-linked leukocyte receptors have been found to be leukocyte integrins (CR3 and CR4) [12–22]. Thus, membrane signaling involves both lateral and transmembrane mechanistic steps (Fig. 1).

In this paper we will review studies focusing on the role of leukocyte integrins as public transducers for a heterogeneous variety of private membrane receptors, including GPI-linked receptors. Although this field is rapidly expanding, CR3 has already been shown to act as a public transducer for numerous private receptors (Table 1). In addition to its broad public functions, CR3 also pos-

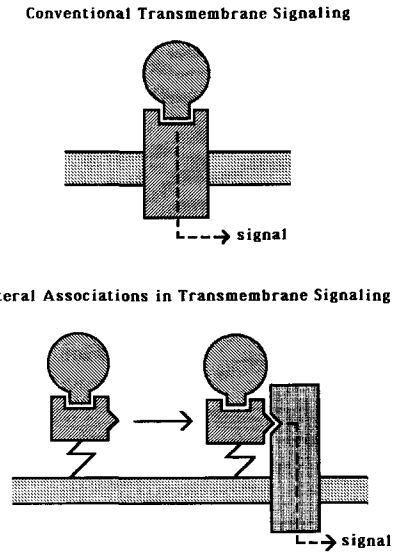

Fig. 1. A schematic illustration of transmembrane signaling mechanisms. During conventional transmembrane signaling a ligand binds to a receptor to elicit a signal (top). However, certain GPI-linked proteins signal by first binding to a transmembrane protein (bottom). In this second mechanism, lateral interactions among membrane proteins are believed to play a crucial role.

Table 2
Experimental tools to study lateral receptor interactions

Methods	Example(s)
Co-capping	[14,18]
Resonance energy transfer	[15,18,21,22]
Fluorescence photobleaching recovery	[15]
Immunoprecipitation	[3,20]
Flow cytometry	[27,43]
Genetic complementation	[76,81]

sesses broad private recognition abilities including the ability to bind iC3b, fibrinogen, factor X, intercellular adhesion molecule 1 (ICAM-1), β-glucans, zymosan, *E. coli*, and *Leishmania* [23]. These multiple specificities can be accounted for by the fact that many of these components (GPI-linked receptors, β-glucans, zymosan, *E. coli* and others) may interact with the same lectin-like site on CR3. In this article we will focus on the ability of CR3 to bind other receptor molecules, not exogenous molecules or organisms.

2. Experimental tools

A variety of experimental tools have been utilized to detect lateral associations of membrane receptors. These tools include cell biological, biophysical, biochemical and genetic methods (Table 2). Early experiments employed receptor co-capping to detect receptor–receptor associations [12,14]. Capping studies remain a useful method to screen for these interactions. More sophisticated experiments relying upon resonance energy transfer (RET) between membrane-associated chromophores [15,18–21] have been utilized to detect lateral proximity relationships between receptors in cell membranes. Fluorescence recovery after photobleaching (FRAP) can be used to detect receptor–receptor interactions if one of the receptors rapidly diffuses in cell membranes (e.g. a GPI-linked protein) and the second receptor (e.g. an integrin) is a large slowly diffusing membrane protein. When a GPI-linked protein becomes tethered to a large slowly diffusing membrane protein, it assumes the smaller diffusion coefficient of its membrane partner; thus providing a means to detect their interaction [15]. The great advantages of co-capping, RET and FRAP experiments over other experimental techniques are: (1) intact, living cells are analyzed, thus avoiding potential artifacts due to cell lysis and solubilization, and (2) studies can be performed in real time, thus revealing the dynamic aspects of receptor–receptor interactions. Flow cytometry methods have been used to detect the binding of soluble receptors to leukocyte integrins. Recently, mild immunoprecipitation experiments have been used to co-precipitate uPAR with various integrin classes. In addition to these methods, traditional genetic complementation experiments, reverse genetic complementation experiments relying upon site-directed mutagenesis studies and cotransfection, and chimeric receptor constructs have been employed to test for receptor–receptor cooperation. A key point here is that receptor–receptor interactions have been verified using a broad battery of scientific tools. We will now discuss specific receptor–receptor interactions, focusing largely on GPI-linked membrane receptors.

3. Static and dynamic associations of GPI-linked proteins with integrins

Dozens of GPI-linked membrane proteins have been shown to elicit transmembrane signals [12–22]. This, of course, raises a fundamental paradox: how can receptors lacking transmembrane segments elicit transmembrane signals? In this section we will explore the role of integrins as public transducers for private GPI-linked receptors.

3.1. FcγRIIIB–β_2 integrin interactions

FcγRIIIB is an extensively glycosylated GPI-linked membrane protein ($M_r \approx 50$–70 kDa) [24,25]. FcγRIIIB triggers cytoplasmic calcium changes, degranulation, actin polymerization and IL-6 and IL-8 production [8,9,26,27]. Early studies suggested that complement receptors may cooperate with Fc receptors to effect cell function [28,29]. For example, IgG-dependent phagocytosis

is inhibited by Fab fragments directed against CR3 [29]. Moreover, leukocyte adhesion deficiency (LAD) patients, who lack $\beta 2$ integrins, display aberrant IgG-dependent functions [30,31]. Functional studies have also suggested that FcγRs interact with CR3 [32–35]. Furthermore, ligation of FcγRs with surface attached immune complexes triggered the formation of new cytoskeletal associations between CR3 and microfilaments, not FcγRs and microfilaments [13].

We first proposed that FcγRIIIB physically interacts with CR3 to elicit cellular responses [12,13]. Early experiments showed that FcγRIIIB co-capped with CR3 [13]. Importantly, these studies also indicated that co-capping could be specifically inhibited by certain saccharides, including *N*-acetyl-D-glucosamine and α-methyl-D-mannoside. This result suggested a mechanism wherein a carbohydrate chain of FcγRIIIB binds to CR3. Ross et al. [36,37] had previously shown that CR3 possesses a lectin-like site with this same specificity. Kimberly's group has also shown that FcγRIIIB binds to lectins of similar specificity [38,39]. Although these facts suggested a simplifying new paradigm, one could not rule out the possibilities that another protein 'glued' FcγRIIIB to CR3 or that another transmembrane protein caused FcγRIIIB to co-cap with CR3 via cytoskeletal links.

To better understand FcγRIIIB-to-CR3 interactions, we studied their interaction in the absence of capping stimuli. RET, which is sensitive to the lateral proximity of membrane receptors, was used to detect FcγRIIIB-to-CR3 proximity on living cell membranes. Specific FcγRIIIB-CR3 proximity was detected on neutrophil membranes in the absence of a capping stimulus [40]. To further characterize these interactions, we transfected genes encoding CR3 and FcγRIIIB into a murine fibroblast cell line. Again, RET was observed between FcγRIIIB and CR3 [15]. To provide another means of FcγRIIIB-to-CR3 interaction, we studied the lateral diffusion of FcγRIIIB in the presence and absence of CR3 using fluorescence recovery after photobleaching (FRAP) [15]. These experiments showed that CR3 significantly reduces the mobile fraction of FcγRIIIB in transfectants expressing both receptors. Two experimental lines of evidence suggested that FcγRIIIB and CR3 interact with a 1:1 stoichiometry. This included both pairwise RET experiments and parallel reductions in the mobile fraction as judged by FRAP experiments. This is, of course, consistent with the fact that only one lectin-like site has been associated with CR3. Furthermore, these data allowed estimates of receptor–receptor lateral dissociation constants to be made. Thus, CR3 tethers FcγRIIIB in cell membranes.

Several lines of evidence have indicated that carbohydrates play a central role in FcγRIIIB-CR3 interactions (as well as CR3 interactions with other GPI-linked proteins, see below). As previously mentioned, we have speculated on the basis of saccharide and polysaccharide inhibition data that the lectin-like site of CR3 was responsible for FcγRIIIB binding. Recent mapping studies have localized CR3's lectin-like site and its region which participates in binding to FcγRIIIB to the same vicinity [41,42]. Using a panel of monoclonal antibodies, Stockl et al. [41] found that the C-terminal region of CR3's α_M chain was responsible for FcγRIIIB binding. Furthermore, NADG inhibited the binding of antibody VIM12 to the C-terminal region the α_M chain; thus the binding of VIM12 and FcγRIIIB to CR3 are both inhibited by the hapten sugar NADG. Importantly, CR3's lectin-like site [36,37] has recently been mapped to the C-terminal domain of the α_M chain using a panel of monoclonal antibodies and chimeric integrin subunits [42]. The lectin-like region of CR3 thus plays a crucial role in binding FcγRIIIB.

Further evidence supporting the proposed interactions between FcγRIIIB and CR3 comes from studies using soluble FcγRIIIB (sFcγRIIIB). Using flow cytometric methods, Teillaud et al. [43] showed that sFcγRIIIB specifically binds to human leukocytes. Recent studies by Galon et al. [27] have shown that both native and recombinant sFcγRIIIB molecules bind to CR3 and CR4. Moreover, binding could be inhibited by antibodies directed against specific epitopes of CR3 or specific carbohydrates including NADG and α-methyl-D-mannoside (see below). Thus, a wide variety of experimental designs support the pro-

posed FcγRIIIB-CR3 association and their proposed mechanism of interaction.

Although FcγRIIIB interacts with CR3 in cell membranes, the interaction is not necessarily physiologically relevant. As mentioned above, early evidence for functional cooperation between CR3 and FcγRs came from patients deficient in CR3 and antibody inhibition experiments [29,30], although these experiments did not address the mechanism of receptor cooperation. To address this issue, the signaling and functional activities of FcγRIIIB were explored under conditions which affected FcγRIIIB-to-CR3 interactions. Zhou and Brown [44] have shown that FcγRIIIB and CR3 cooperate with FcγRII in generating a respiratory burst and tyrosine phosphorylation signaling. Previous studies by Kimberly et al. [8] showed that FcγRIIIB in neutrophil membranes is capable of generating a cytoplasmic calcium signal. To determine if lectin-like sites participate in physiological actions, we treated cells with several types of monosaccharides and polysaccharides [16,45]. NADG, mannose and α-methyl-D-mannoside significantly reduced immune complex-induced cytoplasmic calcium signals in neutrophils [16], although a panel of control monosaccharides had no effect. These reagents similarly affect superoxide anion production and cannot be explained as an effect on FcγRII [16]. Furthermore, the anti-CR3 antibody VIM12, whose binding is blocked by NADG, mimics FcγRIIIB-mediated activation (intracellular calcium rise, actin polymerization, and granule component release) [41]. Neutrophils from LAD patients expressed diminished capacity to trigger intracellular calcium signals and superoxide production. Mannan and chitin, which are polymers of mannose and NADG, respectively, were unable to stimulate superoxide production. The polysaccaharides inhibited the production of superoxide by immune complex-stimulated neutrophils, but not PMA stimulated cells [45]. Since these polysaccharides did not inhibit immune complex binding, they may be interferring with FcγRIIIB-to-CR3 binding. Indeed, we and others have shown that certain polysaccharides, especially β-glucans, can compete with GPI-linked proteins for CR3 [27]. Acemannan immunostimulant, an anti-inflammatory polysaccharide composed of ≈75% acetylated mannan, affects β_2 integrin function [46] and may function by a similar mechanism blocking pro-inflammatory GPI-receptor signaling. Thus, there is good evidence supporting a role of CR3's lectin-like site in neutrophil function.

To provide another line of evidence supporting the physiological importance of FcγRIIIB-to-CR3 interactions, we transfected the genes corresponding to these receptors into murine fibroblasts [17]. Cells transfected with CR3 were capable of interalizing β-glucan particles, but were not capable of binding IgG-coated red cells. Cells transfected with FcγRIIIB were capable of IgG-dependent binding, but not phagocytosis. In contrast, cells transfected with both FcγRIIIB and CR3 were capable of both IgG-dependent binding and phagocytosis. This reconstitution study shows that FcγRIIIB can cooperate with CR3 in a well-defined non-hematopoietic system and suggest that these two proteins are sufficient for phagocytic signaling.

3.2. *uPAR–β_2 integrin interactions*

Urokinase-type plasminogen activator (uPA) binds to cell surface uPA receptors (uPAR), which are extensively glycosylated GPI-linked membrane proteins ($M_r \approx 55\,000$) [47–49]. On the exoplasmic side of cell membranes, uPA catalyzes the activation of zymogen plasminogen and latent enzymes such as procollagenases, thus initiating the powerful proteolytic cascade required for cell migration in vivo [50]. uPA is also competent to generate cytoplasmic signals and physiological responses in leukocytes. Cytoplasmic signals include calcium responses and tyrosine phosphorylation [19,51]. uPA is chemotactic and mitogenic for leukocytes [52,53]. As described above for FcγRIIIB, leukocyte integrins play an important role in uPAR-mediated signaling.

The first evidence supporting a physical interaction between uPAR and CR3 came from co-capping and RET studies [18]. These experiments showed that uPAR and CR3 were in close physical proximity on neutrophil membranes and that capping was not a condition for receptor association. Subsequent experiments from other labora-

tories confirmed uPAR–CR3 co-capping [20]. Importantly, Bohuslav et al. [20] developed a CR3 immunoprecipitation protocol that retains uPAR–CR3 interactions. Thus, several independent lines of evidence agree that CR3 and uPAR interact in leukocyte membranes.

CR3–uPAR interactions are not a static, unchanging property of these receptors, but rather are dynamic associations linked with cell activities. During co-capping experiments we noticed that as neutrophils polarize for locomotion, CR3 trafficked to the uropod while uPAR moved to the lamellipodium of the cell [21]; these facts were previously noted in individual labeling studies [54,55]. This phenomenon was observed during several labeling protocols. Quantitative kinetic RET studies provided further evidence that CR3 and uPAR uncouple as cells begin polarization

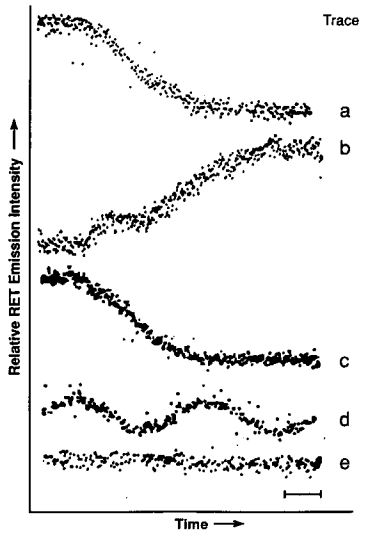

Fig. 2. Kinetic studies of several receptor–receptor interactions. The relative RET intensity is shown at the ordinate; time is plotted at the abscissa. Each receptor type was separately labeled with donor or acceptor chromophores (Table 2). Trace a shows the dissociation of CR3–uPAR complexes as neutrophils begin locomotion. Trace b illustrates the reassociation of CR3 and uPAR as cells stop locomotion. Trace c shows the dissociation of CR3 and lipopolysaccharide-loaded CD14 as cells adhere to a substrate. Panel d shows the oscillatory interaction between CR4 and uPAR on migrating cells. However, when cells are fixed with paraformaldehyde, CR3 and uPAR do not dissociate (trace e). Bar = 30 s for traces a, b, c. Bar = 10 s for trace e.

(Fig. 2a) and reassociate as cells become stationary (Fig. 2b). Thus, CR3 is associated with uPAR on adherent neutrophils, but releases uPAR during cell locomotion. This, of course, raises the issue of how uPAR, a GPI-linked protein, is restrained at the lamellipodium during cell migration. This question is at least partially answered by the homologous integrin CR4, which accumulates at the lamellipodium during locomotion. RET experiments have shown that CR4 and uPAR are in close physical proximity on migrating neutrophils; however, the RET signal does not reach a stable level, but rather oscillates (Fig. 2d) [56]. These observations have been linked to intracellular metabolic oscillations [56]. (Several other leukocyte functions such as actin assembly and oxidant production also oscillate with the same period [57,58].) Thus, CR3 and CR4 contribute to the neutrophil's axis of polarity and reversibly interact with uPAR, depending upon physiological conditions.

The physiological importance of integrin–uPAR interactions on leukocyte membranes has found support from several standpoints. The microscopic studies reported above unambiguously link specific integrin–uPAR associations with cell adherence or locomotion. For example, CR4 focuses uPAR at the lamellipodium of migrating neutrophils [59]; in parallel, pericellular proteolytic activity is also focused at the lamellipodium (unpublished). In additional functional studies, intact uPA was found to heighten intracellular calcium levels in human neutrophils [19]. Two lines of evidence suggest that these calcium changes require CR3: (1) LAD neutrophils do not exhibit increased calcium levels in the presence of exogenous uPA; and (2) transfection of both uPAR and CR3, but not either receptor alone, leads to a uPA-mediated calcium sensitivity in fibroblasts [19]. Although uPA does not trigger superoxide production by neutrophils, it does prime neutrophils for superoxide production; thus the dose-response curve for *N*-formyl-met-leu-phe-mediated superoxide release is shifted almost 100-fold toward lower doses [19]. uPAR antisense oligonucleotide treatment reduces chemotaxis [59]. Similarly, specific saccharides affecting CR3–uPAR interactions also block chemotaxis [60],

suggesting that these interactions play a fundamental role in leukocyte motility. In addition to CR3's role as an effector for uPAR, uPAR can regulate CR3's private adherence function [61]. Monocyte treatment with antisense uPAR oligonucleotides reduces CR3-dependent, but not CR3-independent, adhesion. Thus, the β_2 integrin–uPAR collaboration plays an important role in leukocyte function.

3.3. uPAR–β_1, β_3 integrin interactions

Just as uPA–uPAR complexes are required for leukocyte migration, uPA–uPAR complexes are required for tumor cell metastasis [62]. uPA and uPAR are known to cluster at sites of tumor cell adherence [63–65]. Since integrins also cluster at adherence sites [1], we tested the hypothesis that tumor cell integrins interact with uPAR. Using co-clustering, RET, and β_1 integrin immunoprecipitation techniques, we have shown that certain integrins are associated with uPAR when fibrosarcoma cells are adherent to specific extracellular matrix components [66]. Cell adherence to fibronectin, laminin, and vitronectin, but not polylysine, leads to β_1 integrin–uPAR association. However, only vitronectin triggered β_3 integrin–uPAR proximity. α chain specificities were consistent with the $\alpha_5\beta_1$ fibronectin receptor and the laminin and vitronectin receptors as uPAR partners [66]. Saccharide-mediated inhibition of co-clustering and RET were noted, suggesting that extracellular matrix-inducible lectin-like integrin–uPAR interactions may participate in regulating the spatial distribution of pericellular proteolysis of tumor cells.

3.4. CD14–β_2 integrin interactions

CD14 is another GPI-linked protein expressed on leukocytes [67]. When lipopolysaccharide (LPS)–lipoploysaccharide binding protein (LBP) complexes interact with CD14, transmembrane signals including tyrosine phosphorylation of specific proteins and NF-kB translocation to the nucleus are observed [68,69]. From a physiological standpoint, CD14 participates in: (1) cytokine production; (2) cell adherence; and (3) non-opsonized internalization of bacteria [69–71].

Integrins may participate in some forms of CD14-mediated physiological signaling [69,70]. We have recently shown that LPS and LBP trigger an association between CD14 and CR3 [22]. However, the CD14–CR3 interaction rapidly disappeared as neutrophils adhered to substrates (Fig. 2, trace c). As previously stressed for uPAR, the CD14–CR3 interaction is transient. This interaction may transiently increase CR3's avidity for substrates thus potentiating cell adherence. CR3 (and/or CR4) may also participate in non-opsonized internalization of bacteria. Preliminary studies in this laboratory have shown that NADG and β-glucan, but not a panel of unrelated saccharides, are capable of reducing or eliminating the non-opsonized phagocytosis of *E. coli*. In the case of the monosaccharide NADG, a high degree of phagocytic inhibition ($\approx 80\%$) was achieved in the absence of any affect on the total number of bacteria bound. This suggests that integrins may play a role in non-opsonized particle internalization. Since LPS can also interact with CR3 and CR4 [69,72], potential mechanisms involving a direct physical interaction between receptors during phagocytosis or the crosslinking of CD14 to integrins via LPS-containing bacterial membranes are possible. However, soluble LPS–LBP complexes can trigger physical interactions between CD14 and CR3.

4. Non-GPI-linked receptors can also associate with β_2 integrins

In the preceding paragraphs we stressed the ability of GPI-linked receptors to physically and functionally interact with integrins. However, CR3's receptor–receptor interactions extend beyond GPI-linked membrane proteins. These additional CR3-binding receptors are all Fc receptors: FcγRII (CD32), soluble FcγRIIIB and FcεRII (CD23).

As mentioned above, the ability of Fc receptors to cooperate with complement receptors has been well-known. The ability of FcγRIIIB to physically interact with CR3 [12–17] suggested that other FcRs may physically interact with complement receptors. Using K562 cells, which express

FcγRII, CR3 and CR4, Annenkov et al. [73] showed that anti-FcγRII antibodies specifically inhibit CR3-mediated cell adhesion. Functional interactions of FcγRII with CR3 and FcγRIIIB have been suggested in the respiratory burst and tyrosine phosphorylation signaling [44]. Physical proximity between FcγRII and CR3 in neutrophil membranes has been demonstrated in preliminary studies [40]. Functional cooperation between FcγRII and CR3 has also been observed in eosinophil membranes, which do not express other FcγR classes. In these studies presentation of FcγRII and CR3 ligands in close physical proximity elicited synergistic functional responses [74,75]. Functional cooperation beween FcγRII and CR3 has been demonstrated in transfectants. Reverse genetic complementation studies were performed using a phagocytosis-defective tail-minus form of FcγRIIA [76]. Although this defective FcγRIIA-tail mutant was unable to mediate IgG-dependent phagocytosis in transfectants [76,77], co-transfection with CR3 was sufficient to trigger antibody-dependent phagocytosis [76]. RET experiments showed close physical proximity between CR3 and FcγRIIA in transfectant membranes, suggesting that the mechanism of complementation apparently involves physical contact between these two receptors. However, this mechanism differs from those presented above since a role for the lectin-like site of CR3 has not been observed. Thus, FcγRII, a transmembrane protein, can interact with CR3 in leukocyte and transfectant membranes.

Soluble forms of FcRs have been observed in vitro and in vivo. As briefly mentioned above, sFcγRIIIB binds to CR3 and CR4 to elicit cytokine production [27]. sFcεRII, a low affinity receptor for IgE, interacts with CR3 and CR4 of monocytes to elicit cytokine and oxidant production [78]. Thus, soluble forms of FcRs may play a crucial role in regulating inflammatory responses via their interaction with leukocyte integrins.

5. Inter-receptor dysfunction disorder

We have recently identified a patient diagnosed with pyoderma gangrenosum who has exhibited skin ulceration due to mild bruises throughout her 14 year lifetime; on one occasion she exhibited a septic shock-like disorder without sepsis. Neutrophils from this patient fail to polarize and migrate normally in vitro. Abnormal migration in vivo was also demonstrated using skin window assays. Neutrophils from this patient display highly clustered β_2 integrins and aberrant oxidant production. Concommitantly, CR3 interactions with FcγRIIIB and uPAR are significantly reduced, whereas CD14-CR3 interactions are increased (unpublished). Occult bacterial infection has been clinically ruled out as a potential stimulant source. Furthermore, addition of serum from the patient or LPS to normal neutrophils failed to reconstitute the observed cellular defects. Preliminary studies suggest that this may be due to an aberrant form of CD18, although molecular biological analyses are currently underway. We have found that cooling cells to 34°C returns CR3 to its normal distribution and allows normal neutrophil polarization and migration in vitro. Local hypothermia has been found to be effective in managing skin involvement.

6. Biochemical survey of CR3 membrane associations

As mentioned above, it is now possible to preserve inter-receptor interactions during immunoprecipitation using the mild detergent Brij-58 [20,66]. To inventory membrane proteins interacting with CR3, we biotinylated cell membranes followed by extraction using Brij-58 as described by Bohuslav et al. [20]. Fig. 3 shows representative results after SDS-PAGE and Western blotting using avidin reagent. In addition to bands representing the α and β chains of CR3, bands of approximate $M_r = 40\,000$, $50\,000$, $74\,000$, and $120\,000$ were observed. Notably, cytoplasmic actin is absent from these luminograms. The diffuse band at $\approx 50\,000$ contains uPAR, as shown by Western blotting (unpublished). Thus, several membrane proteins interact with CR3 in cell membranes. Several previous studies have identified cytoplasmic proteins (e.g. p16, $p60^{fyn}$, $p53/56^{lyn}$, $p58/64^{hck}$, and $p59^{fgr}$) that are associated

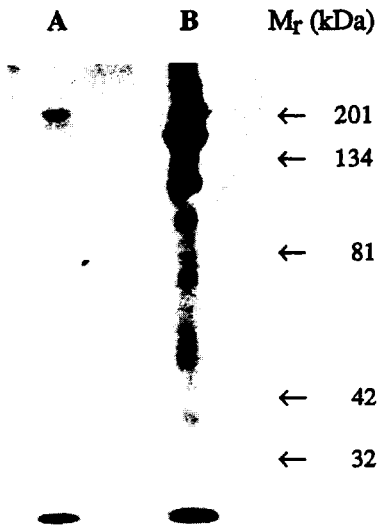

Fig. 3. Analysis of membrane proteins co-immunoprecipitating with CR3 from human neutrophils. Neutrophil membrane proteins were labeled with a membrane-impermeable biotin reagent. Cells were then lysed and immunoprecipitated using a solution containing Brij-58. After SDS-PAGE, samples were analyzed by Western blotting using an avidin–peroxidase conjugate. In the absence of specific anti-CR3 antibody, only one non-specific band is observed (lane A). In the presence of specific anti-CR3 antibody, both integrin subunits and several additional proteins are found (lane B). Thus, the mild detergent Brij-58 preserves multiple protein–protein interactions during immunoprecipitation.

with CR3-containing membrane complexes [20,79].

7. Comparison with other lateral membrane associations in signal transduction

Lateral interactions between membrane proteins to mediate signaling are not limited to integrins. Indeed, in recent years lateral interactions have been discovered in a variety of signal transduction systems. A public transducer system similar to integrins was identified in cytokine and neurokine signal transduction (Table 1). Kishimoto and others showed that gp130 is a public transducer for receptors recognizing IL-6, IL-11, oncostatin M, leukemia inhibitor factor, and ciliary neutrotrophic factor (CNTF) [2–4,80–82]. Interestingly, the CNTF receptor is a GPI-linked membrane protein [83]. The gp130 public transduction system, however, differs from the integrin public transduction system since the β_2 integrins' private receptor counterparts (e.g. FcγRIIIB, uPAR, etc.) bind their ligands with the same affinity in the presence and absence of integrins. Public transduction of physiological signals in the presence of IL-6 were observed for the wild-type IL-6 receptor, a truncated form lacking the cytoplasmic domain, and a soluble form of the receptor [3]. Thus, public transducers such as gp130 and integrins may be of broad importance in cell function.

Lateral interactions in signaling, however, are broader than the private receptor–public transducer paradigm. For purposes of comparison, we have classified these additional lateral interactions as: (1) composite receptor signaling mechanisms and (2) lateral exchange mechanisms (Table 3, Fig. 4). These systems are much too complicated to review in detail here and references to the literature can be found in Table 3. Our purpose here is to briefly compare strategies used for lateral membrane signaling. In composite receptor mechanisms, the affinity of a receptor for a ligand could be below the physiologically-relevant region, but is increased by the presence of a second membrane protein. For example, the human IL-5

Table 3
Examples of composite and lateral exchange interactions in membrane signaling

Ligand	Recognition and transduction elements	Reference(s)
A. Composite signaling apparatus		
IL-2	IL-2R α, β, γ	[93]
TGF-β	TGFβ-RII, TGFβ-RI	[85]
Heregulin	erbB2, erbB3, erbB2, erbB4	[94]
IL-3	IL-3R, KH97	[4]
IL-5	IL-5R, KH97	[4,84]
GM-CSF	GM-CSF, KN97	[4]
B. Lateral exchange mechanism		
TNF	$TNFR_{80}$, $TNFR_{60}$	[86]
LRP-uPA·PAI-1	uPAR, LRP	[87,88]

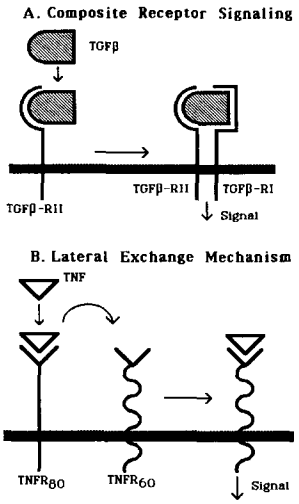

Fig. 4. Schematic illustrations of other types of lateral membrane interactions in signaling. In panel A the signaling mechanism of the TGFβ receptor is illustrated. This composite receptor signaling mechanism requires the presence of a ligand to complete the assembly of a functioning receptor. Panel B shows the lateral exchange mechanism of transmembrane signaling for the TNF receptors. In this mechanism the ligand first binds to one plasma membrane component which then transfers the ligand to a second receptor which elicits a transmembrane signal.

receptor is of low affinity unless it is co-expressed with its β chain, KH97 [84]. Another slightly different example of a composite receptor system is the TGF-β (transforming growth factor-β) signaling apparatus. TGF-β is recognized by two receptors (TGF-β-RII and TGF-β-RI). TGF-β binds to TGF-β-RII which then forms a ternary complex with TGF-β-RI. The intracellular domains of both TGF-β-RI and TGF-β-RII then participate in signal transduction [85]. In this case no public transducer is involved; signal transduction is mediated by the physical and functional cooperation of two private membrane receptors. The second additional class of lateral membrane interactions in signaling is lateral exchange mechanisms. In these cases ligands are transferred from one membrane receptor to a second membrane receptor. One example of this type of mechanism is illustrated by TNF receptors. Cells express two forms of TNF receptors ($TNFR_{80}$ and $TNFR_{60}$). As illustrated in Fig. 4, TNF first binds to $TNFR_{80}$. TNF is then transferred to $TNFR_{60}$ which mediates signal transduction [86]. Another example of a lateral exchange mechanism is the exchange of uPA·PAI-1 (plasminogen activator inhibitor-1) complexes from uPAR to LRP (lipoprotein receptor-related protein) to mediate internalization [87,88]. Thus, lateral membrane interactions leading to physiological processes have been observed in many receptor systems.

8. Conclusions

Studies in our laboratory and many others have identified novel sets of lateral membrane interactions. These lateral protein–protein interactions have been shown to play a crucial role in the transduction of signals across biological membranes (Tables 1 and 3).

Our studies of GPI-linked protein signal transduction apparently resolve the long-standing problem of how GPI-linked proteins elicit transmembrane signals and physiological responses in neutrophil membranes. The integrin system is unique since its mechanism of interaction with GPI-linked proteins, which involves CR3's lectin-like site has been identified. Thus, it is possible to direct rational drug therapy at this molecular site. Although we have shown integrin–uPAR interactions in tumor cell membranes, these integrin interactions have not yet been shown to be a general property of eukaryotic cells. The lateral interactions of integrins (and potentially other systems as well) may provide useful new sites for rational drug development. Indeed, several previously described compounds including indomethicin [56], β-glucan and its fragments [89], and acemannan [46] may influence inter-receptor interactions of integrins [56]. Molecules such as β-glucan (and related materials) and acemannan affect leukocyte function and receptor–receptor interactions by binding to the lectin-like site of integrins. Compounds that act as agonists at this site may be useful in enhancing host resistance to tumors [89], as has already been clinically shown for β-glucans [90–92]. Compounds that act as antagonists may be useful in controlling inflammatory processes during arthritis, septic shock, ischemia-reperfusion injury

and other conditions. Since these interactions, which have only recently been discovered, have already been shown to: (1) function at the cellular level; (2) be affected in a pyoderma gangrenosum patient; and (3) be pharmacologically relevent, they are likely to have long-term clinical relevance.

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