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REVIEW

Function, structure and therapeutic potential of complement C5a receptors

PN Monk[1], A-M Scola[1], P Madala[2] and DP Fairlie[2]

[1]*Academic Neurology Unit, School of Medicine and Biomedical Science, University of Sheffield, Sheffield, UK and* [2]*Centre for Drug Design and Development, Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland, Australia*

Complement fragment (C)5a is a 74 residue pro-inflammatory polypeptide produced during activation of the complement cascade of serum proteins in response to foreign surfaces such as microorganisms and tissue damaged by physical or chemical injury. C5a binds to at least two seven-transmembrane domain receptors, C5aR (C5R1, CD88) and C5L2 (gpr77), expressed ubiquitously on a wide variety of cells but particularly on the surface of immune cells like macrophages, neutrophils and T cells. C5aR is a classical G protein-coupled receptor that signals through Gαi and Gα16, whereas C5L2 does not appear to couple to G proteins and has no known signalling activity. Although C5a was first described as an anaphylatoxin and later as a leukocyte chemoattractant, the widespread expression of C5aR suggested more general functionality. Our understanding of the physiology of C5a has improved significantly in recent years through exploitation of receptor knockout and knockin mice, C5 and C5a antibodies, soluble recombinant C5a and C5a analogues and newly developed receptor antagonists. C5a is now also implicated in non-immunological functions associated with developmental biology, CNS development and neurodegeneration, tissue regeneration, and haematopoiesis. Combined receptor mutagenesis, molecular modelling, structure-activity relationship studies and species dependence for ligand potency on C5aR have been helpful for identifying ligand binding sites on the receptor and for defining mechanisms of receptor activation and inactivation. This review will highlight major developments in C5a receptor research that support C5aR as an important therapeutic target. The intriguing possibilities raised by the existence of a non-signalling C5a receptor are also discussed.
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Keywords: complement; C5a; G protein; receptor; inflammation; immunity; antagonist

Abbreviations: AMD, age-related macular degeneration; C5aR, C5a receptor; Cha, cyclohexylalanine; CREB, cAMP response element-binding; ECL, extracellular loop; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; IC, immune complex; IFN, interferon; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MAPKAP-K2, MAPK-activated protein kinase 2; PAK, p21-activated kinase; PMN, polymorphonuclear leucocyte; PT, pertussis toxin; RA, rheumatoid arthritis; RSM, random saturation mutagenesis; SMC, smooth muscle cells; SNP, single-nucleotide polymorphism; STAT3, signal transducers and activators of transcription; TM, transmembrane; TNF, tumour necrosis factor; Trp5, *N*-MethylPhe-Lys-Pro-*D*Cha-Trp-*D*Arg-CO_2H.

Formation of C5a

Human complement is a complex network of soluble and membrane-associated serum proteins that form a highly regulated, exquisitely directed and normally measured humoral and cellular immune response to infectious organisms (bacteria/viruses/parasites), to tissue damaged by chemical, physical, radiation or neoplasia insults and to other foreign surfaces not recognized as 'self'. The complement system is an ancient part of the innate immune system that has existed and adapted for >350 million years, components having been identified in organisms as primitive as the Horseshoe Crab (*Carcinoscorpius rotundicauda*) (Zhu *et al.*, 2005). After encountering pathogen-associated molecular patterns, activation of the complement system proceeds through a stepwise hierarchy of proteolytic activation events, each proteolytic enzyme catalytically cleaving downstream members of the cascade.

Complement activation occurs through three different pathways (Figure 1). The *classical* activation pathway, initially described as a 'complement' to specific antibody lysis of bacteria (Bordet, 1895), is a response to the formation of immune complexes (IC) of complement fixing IgG1 and IgM antibodies. More recently, low affinity IgM antibodies involved in defence against infection and cancer and

Correspondence: Dr PN Monk, Academic Neurology Unit, School of Medicine and Biomedical Science, University of Sheffield, Beech Hill Road, Sheffield S10 2RX, UK.
E-mail: p.monk@shef.ac.uk
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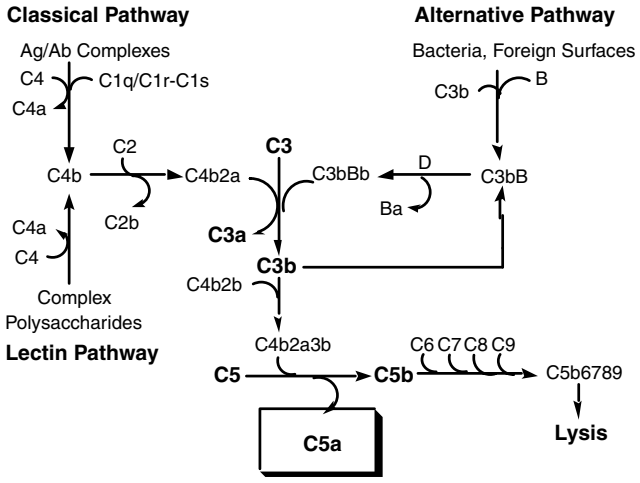

Figure 1 Three pathways for complement activation.

increasingly recognized as an important part of the innate immune system have also been shown to activate complement through the classical pathway (Vollmers and Brandlein, 2006). A second *lectin* activation pathway is initiated by lectins, which recognize the sugar structures that decorate the surfaces of infectious organisms. The third activation mechanism is the *alternative* pathway, which relies on the continuous degradation of component C3 that occurs on pathogen and host cell surfaces. Further complement activation is usually inhibited by control factors such as decay-accelerating factor and CD59 but the lack of these control factors on 'non-self' surfaces leads to a rapidly amplified complement cascade activation (Thurman and Holers, 2006). In primitive organisms, the complement cascade is primarily opsonic, leading to the phagocytosis of targets. In higher organisms such as mammals, there are more than 30 serum components in the cascade, reflecting the complex effector pathways that lead not just to opsonization but also to the formation of a lytic membrane attack complex that perforates membranes of microorganisms causing cell death. Among other products are small protein anaphylatoxin fragments C3a, C4a and C5a.

Each of the three pathways produces C5a and C5b, the latter assembling with C6, C7, C8 and C9 serum proteins to form the membrane attack complex. The cascade is highly regulated to avoid stepwise amplification but uncontrolled or aberrant regulation, resulting in protracted complement activation, can cause disease. Serum is a reservoir of the precursors of the complement fragments and so, even in the early stages of the innate immune response, high concentrations of these fragments may be produced and sustained for prolonged periods. Unlike C3a, for which even resting concentrations are high (>100 nM) because of the continual degradation of C3, there is almost no detectable C5a in the resting state (<1 nM) of healthy individuals. After activating human serum with cobra venom factor, concentrations of C5a can reach $\sim$285 nM. Interestingly, complement fragments can also be directly generated by proteases unrelated to the complement cascade; C5 degradation by thrombin, a participant in the coagulation cascade, causes C5a production even in animals with a genetic deficiency of the upstream complement protein C3 (Huber-Lang *et al*., 2002). Similarly, proteases found in allergenic house dust mite (*Dermatophagoides farinae*) faeces have been shown to generate anaphylatoxins from purified human C3 and C5, suggesting a possible route to C5a (and C3a) production in asthma (Maruo *et al*., 1997). Pro-inflammatory amorphous silica (Governa *et al*., 2005) and asbestos fibres (Governa *et al*., 2000) have also been shown to activate C5 directly.

This review focuses on the function, structure and therapeutic potential of the cell surface receptors for one of these human complement fragments, namely C5a.

Structure and function of C5a

Human C5a (12–14.5 kDa) is composed of 74-amino acids, including Asn64, which has an N-linked carbohydrate moiety that is not essential for biological activity but very likely regulates C5a activity *in vivo*. It is missing from the highly homologous (69%) but equipotent porcine C5a. The solution structure (Zhang *et al*., 1997; Zuiderweg and Fesik, 1989; Zuiderweg *et al*., 1989) of human C5a (Figure 2) shows an antiparallel 4-helix bundle (residues 1–63), the four different helical segments (4–12, 18–26, 32–39, 46–63) being stabilized by three disulphide bonds (Cys_{21}-Cys_{47}, Cys_{22}-Cys_{54}, Cys_{34}-Cys_{55}) and connected by loop segments 13–17, 27–33 and 40–45. The 63-residue helix bundle fragment is highly cationic and confers high affinity for the cell surface. The C-terminal residues 69–74 also form a bulky helical turn connected to the 4-helix bundle by a short loop. Reducing disulphide bonds or selectively removing residues before the N-terminal disulphide from C5a1 to 74 substantially decreases function. The fragment C5a1–69 missing the C-terminal pentapeptide binds to cells but has no agonist activity, consistent with the N-terminal helix bundle conferring affinity, while the C-terminus alone is the receptor-activating domain. Loop 1 (residues C5a12–20, including four Lys residues 12, 14, 19, 20), loop 3 (C5a39–46) and the C-terminal 6–8 residues (especially Arg74) are important for binding to C5a receptor (C5aR) and agonist potency. Neutralizing antibodies to C5a have implicated the region Lys20-Arg37 as important for receptor binding.

C5a is readily metabolized by serum and cell-surface carboxypeptidases (Bokisch and Muller-Eberhard, 1970) that remove the C-terminal arginine to form 'C5a des Arg', reducing potency to only 3–10% for promoting neutrophil chemotactic activity and to $<1\%$ in inducing a spasmogenic response from ileal tissue. Further removal of the C-terminal pentapeptide by carboxypeptidase Y inactivates the molecule ($<1\%$) for both chemotactic and spasmogenic activity. The enzyme-linked immunoadsorbent assays used to measure serum C5a detect both forms equally well. The high activity levels of carboxypeptidases mean that most, if not all, of the C5a detected is actually C5a des Arg.

C5a was first described as a classical anaphylatoxin, capable of stimulating the secretion of histamine from mast cells (Friedberger, 1910), and later identified as a potent neutrophil (Snyderman *et al*., 1970; Becker, 1972) and macrophage (Snyderman *et al*., 1975) chemoattractant. Now C5a is recognized as a pleiotropic molecule that can

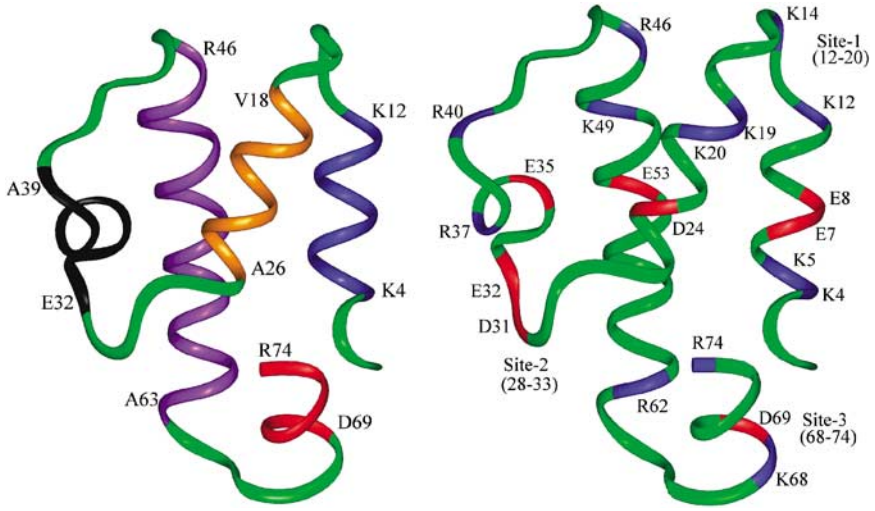

Figure 2 Solution structure of human C5a determined from 1H NMR spectroscopy in H_20/D_2O (Zhang *et al.*, 1997), showing (left) : four helices $MLQK_4KIEEIAAK_{12}YKH$ (blue), $SVV_{18}KKCCYDGA_{26}CVNN$ (orange), $DE_{32}TCEQRAA_{39}RISLGP$ (black), $R_{46}CIKAFTQCCVVA_{63}SQ$ (violet) joined by loops (green) and a C terminal $D_{69}GLGR_{74}$ (red), which adopts a 1.5 turn helix joined to the four helix bundle by a short loop (green); (right): electrostatic map showing residues with charged acidic (red) or basic (blue) side chains.

modulate the activity of many cell types, with a broad range of biological functions both inside and outside of the immune system. All cells of the myeloid lineage, including eosinophils (Kay *et al.*, 1973), basophils (Hook *et al.*, 1975) and neutrophils, sub-populations of monocytes (Falk and Leonard, 1980) and most, if not all, tissue macrophage types (including alveolar macrophages (McCarthy and Henson, 1979), liver Kuppfer cells (Laskin and Pilaro, 1986) and microglia in the central nervous system (Yao *et al.*, 1990)) respond to C5a.

Although some types of lymphoid cells have been shown to respond to C5a, this is not universally accepted. Both B and T lymphocytes were initially reported to migrate towards C5a (El-Naggar *et al.*, 1980); helper T cells were shown to have an increased antigen-induced proliferative response in the presence of C5a (Morgan *et al.*, 1983) and germinal centre (Kupp *et al.*, 1991), and naïve tonsillar (Ottonello *et al.*, 1999) B cells migrate in response to C5a. In contrast, a study using fluorescently labelled C5a failed to show significant binding to more than 6% of lymphocytes (van Epps and Chenoweth, 1984), and anti-C5aR antibodies did not bind to murine lymphoid cells (Soruri *et al.*, 2003b). However, low levels of the C5aR have been detected by flow cytometry on CD3+ human T cells, particularly after lectin stimulation (0.6% rising to 14.4% positive for C5aR) and on the Jurkat T-cell line (Nataf *et al.*, 1999). The same study showed that CD3+ T cells would migrate towards C5a. Thus, it appears that subsets of lymphocytes may be responsive to C5a and this percentage increases following activation. Mast cells also show highly variable responsiveness to C5a; for instance, skin mast cells respond to C5a, whereas lung and intestinal mast cells do not (Lawrence *et al.*, 1987). More recently, expression of a receptor for C5a has been shown to discriminate between mast cell subsets, which also show distinct differences in protease expression, suggesting that C5a responsiveness is programmed into mast cell development (Oskeritzian *et al.*, 2005).

Although C5a has long been known to induce smooth muscle contraction, this has been thought to be secondary to the release of histamine and arachidonic acid-derived mediators (Regal *et al.*, 1983). However, evidence has accumulated to show that C5a may also have direct effects because smooth muscle cells (SMC) have been shown to express low levels of anaphylatoxin receptors (Haviland *et al.*, 1995; Gasque *et al.*, 1998; Zwirner *et al.*, 1999). However, there are no data on the function of C5a in SMC. In liver, hepatic stellate cells have been shown to undergo a small fibrotic response to C5a (Schlaf *et al.*, 2004), and C5a can act as a growth factor in regenerating rat hepatocytes (Daveau *et al.*, 2004). In fact, the absence of C5 or the blockade of C5aR both lead to impairment of liver regeneration, and the reconstitution of C5-deficient mice with C5a can restore this function (Mastellos *et al.*, 2001). Endocrine and folliculostellate cells of the anterior pituitary gland express both forms of C5aR, and C5a stimulates mitogen-activated protein kinase (MAPK) activation in a mouse pituitary cell line (Francis *et al.*, 2005), suggesting a possible role for C5a in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis.

Elevated levels of C5a have been found in the serum of patients with inflammatory disorders. Overexpression or underregulation of C5a is implicated in human and/or experimental models of inflammatory conditions, such as rheumatoid arthritis (RA) (Grant *et al.*, 2002; Woodruff *et al.*, 2002) and osteoarthritis, adult respiratory distress syndrome (Hammerschmidt *et al.*, 1980b), inflammatory bowel diseases (Woodruff *et al.*, 2003), lupus, ischaemia/reperfusion injury (Arumugam *et al.*, 2003; Martin *et al.*, 1988; Proctor *et al.*, 2004; Woodruff *et al.*, 2004), chronic obstructive pulmonary disease (Marc *et al.*, 2004), sepsis (Huber-Lang *et al.*, 2002), IC disorders (Strachan *et al.*, 2000, 2001) and peritonitis (Godau *et al.*, 2004), asthma and allergy (Abe *et al.*, 2001; Baelder *et al.*, 2005; Gerard and Gerard, 2002; Lambrecht, 2006), psoriasis (Kapp and Schopf, 1985), gingivitis (Okada

and Silverman, 1979), atherosclerosis (Hammerschmidt *et al*., 1981), tissue rejection (Gaca *et al*., 2006), extracorporeal bypass (Tofukuji *et al*., 1998), glomerulonephritis (Kondo *et al*., 2001), meningitis, pancreatitis (Bhatia, 2002), fibrotic conditions (Hillebrandt *et al*., 2005; Jones *et al*., 1998), lung injury (Mulligan *et al*., 1996), neurodegeneration and macular degeneration (Kijlstra *et al*., 2005; van Beek *et al*., 2003), cystic fibrosis (Fick *et al*., 1986), fetal rejection (Girardi *et al*., 2006), systemic lupus erythematosus (Hammerschmidt *et al*., 1980a), anaphylactic and haemorrhagic (Harkin *et al*., 2004) shock, and following major trauma (Sewell *et al*., 2004), burns (Piccolo *et al*., 1999) and infection. Excessive complement activation may thus affect many hundreds of millions of people. Bioavailable C5aR antagonists could conceivably have potent anti-inflammatory properties in many diseases, while agonists could be valuable immunostimulants, enhancing humoral and cellular immunity.

Receptors for C5a

The first human C5aR was cloned in 1991 (Boulay *et al*., 1991; Gerard and Gerard, 1991). The second human C5aR, C5L2, was identified in 2000 (Ohno *et al*., 2000). Both genes are localized to the same region of chromosome 19, q13.33 and encoded in a two exon structure, with the 5′ untranslated region and initiating codon in the first exon, and the remainder of the coding sequence and the 3′ untranslated region in the second (Gerard *et al*., 1993). This is typical of the members of the chemoattractant receptor family. The sequences of C5aR and C5L2 are shown in Figure 3. C5aR is categorized in the peptide receptor subfamily of class A rhodopsin-like receptors. In a recent analysis based on the sequences of the transmembrane (TM) helices (Surgand *et al*., 2006), C5aR and C5L2 clustered with other chemoattractant receptors, such as type-II angiotensin-II receptor, bradykinin receptors, the formyl peptide receptor family, ChemR23 and several orphan G-protein-coupled receptors (GPCRs). Similarly, Joost and Methner (2002) placed C5aR and C5L2 in the GPCR family A8, with formyl peptide receptors, ChemR23 and the orphan receptors GPR1, 15 and 44 based on the sequences from the TM regions. A small number of single-nucleotide polymorphisms (SNP) have been found in both receptor genes. In the promoter region of C5aR, an SNP at position −245 (T/C) has been discovered (Barnes *et al*., 2004) and the coding region C5aR has two non-synonymous SNP at 4G/A (Asp/Asn at amino-acid position 2) and 859G/T (Asn/Lys at position 278) and two synonymous SNP: 72T/C, 727G/A (Birney *et al*., 2006). C5L2 has two synonymous SNP at 614G/A and 860C/T (Birney *et al*., 2006). There are no known associations between these SNP and human disease. Mice in which either C5aR (Hopken *et al*., 1996) or C5L2 (Gerard *et al*., 2005) has been genetically deleted are fully viable, but show alterations in many of the disease processes that involve C5a. The deletion of C5aR demonstrated a non-redundant role for this receptor in mucosal defence (Hopken *et al*., 1996) and in one model of RA (Ji *et al*., 2002) and a role in the reverse passive Arthus response (Hopken *et al*., 1997), contact sensitivity (Tsuji *et al*., 2000), glomerulonephritis

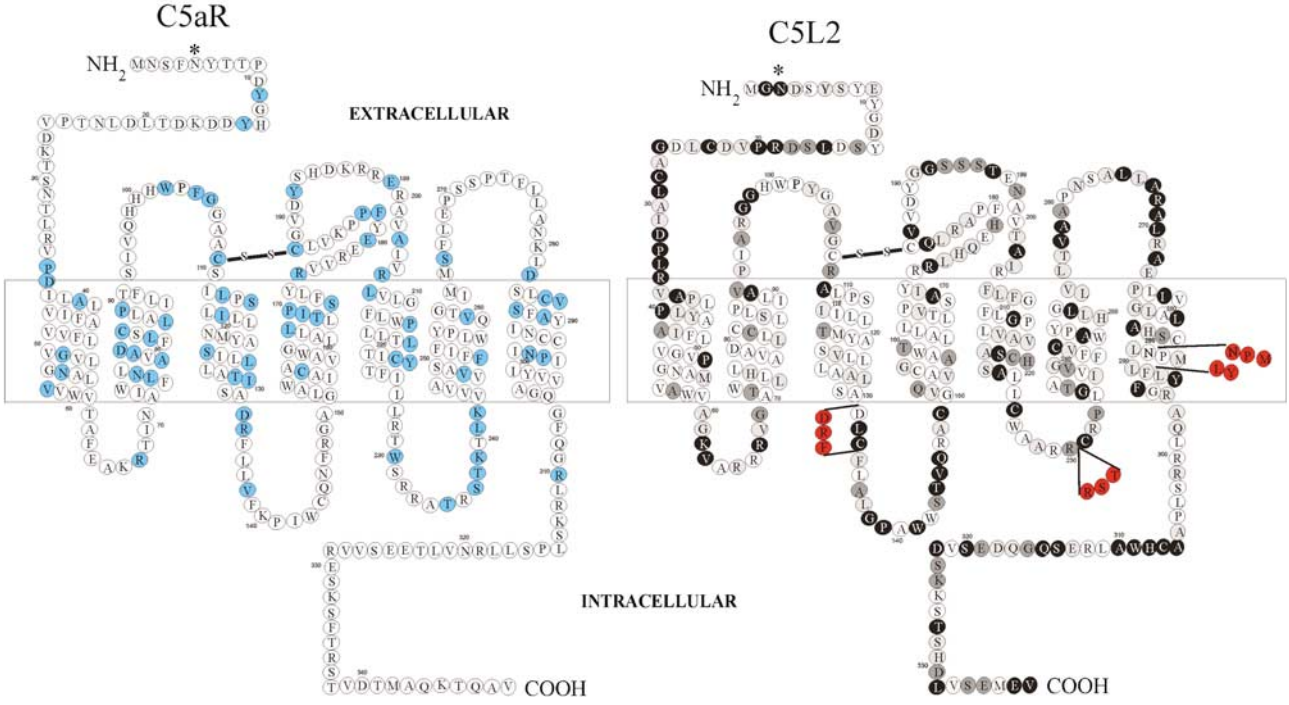

Figure 3 Sequences of the C5a receptors, C5aR and C5L2, with potential glycosylation sites asterisked. Both receptors (42–45 kDa) also have characteristic arrays of Asp and Tyr residues at the N-termini; overall sequence identity is 35%. The degree of conservation for individual residues is shown by the depth of shading on the C5L2. Residues identified in site-directed or random saturation mutagenesis studies as having an important role in ligand binding, and/or signalling by C5aR are shown in blue. Sequences critical for G-protein coupling in C5aR, which are changed in C5L2, are shown in red.

(Welch *et al*., 2002), pulmonary hypersensitivity (Shushakova *et al*., 2002), granuloma formation in response to *Mycobacterium* infection (Borders *et al*., 2005) and in mast cell-mediated neutrophil accumulation in peritonitis (Mullaly and Kubes, 2007). In contrast, the course of experimental autoimmune encephalomyelitis was unaltered (Reiman *et al*., 2002) and Th2 cytokines, high serum IgE levels and substantial recruitment of inflammatory cells were actually increased after pulmonary allergen challenge in C5aR-deficient mice (Kohl *et al*., 2006). C5L2 deficiency has been reported to enhance responses to C5a *in vivo* (Gerard *et al*., 2005) but to diminish the responsiveness to C5a of neutrophils *in vitro* (Chen *et al*., 2007), suggesting multiple roles for this receptor.

Control of receptor expression

Although initially thought to be restricted to mast cells and cells of the myeloid lineage, C5aR expression is now known to be widespread (Table 1). Northern blot analysis has shown the range of expression of C5L2 to be broadly similar to that of C5aR. C5L2 is expressed in various cells and tissues, such as astrocytes, neutrophils/macrophages, mast cells, immature dendritic cells, as well as in the brain, lung, heart, kidney, liver, ovary or testis (Gavrilyuk *et al*., 2005; Lee *et al*., 2001; Ohno *et al*., 2000; Okinaga *et al*., 2003; Otto *et al*., 2004). The control of receptor expression at the gene level has not been thoroughly explored, but distinct transcriptional control mechanisms have been shown to occur in murine microglial cells and astrocytes (Martin *et al*., 2006). At the cellular level, the CCAAT box nuclear factor Y binding site (−96) is involved in lipopolysaccharide (LPS)-induced transcriptional upregulation of C5aR in murine macrophages and endothelial cells (Hunt *et al*., 2005) with minor contributions from a GATA site (−298) and a CP2 site (−155). Prostaglandin E2 upregulates C5aR in monocyte-derived dendritic cells (Weinmann *et al*., 2003), whereas interleukin-4 (IL-4) downregulates C5aR expression in monocytes (Soruri *et al*., 2003a). C5aR is upregulated by IL-6 in the lung, liver, kidney and heart of septic rats (Riedemann *et al*., 2003) and in the brain by tumour necrosis factor-α (TNFα) in closed head injury and Listeria infection in mice (Stahel *et al*., 1997, 2000). Monocytic differentiation of HL-60 cells, in the presence of vitamin D3, is associated with increased expression of C5aR (Zahn *et al*., 1997); C5aR is also upregulated in myeloblastic cell lines by dibutyryl-cAMP, phorbol ester and interferon (IFN)γ (Burg *et al*., 1996; Rubin *et al*., 1991). Less is known about the control of C5L2 expression, but total cellular C5L2 expression has been shown to decrease on neutrophils after exposure to C5a (Huber-Lang *et al*., 2005). A single study has reported regulation of C5L2 expression on cell lines: dibutyryl-cAMP and IFNγ induced upregulation of this receptor on U937 and HL-60 cells, but TNFα had no effect. In the epithelial HeLa cell line, constitutive expression of a low level of C5L2 but not C5aR was detected, and treatment with IFNγ and TNFα drastically reduced C5L2expression (Johswich *et al*., 2006). C5aR is known to rapidly internalize after treatment with C5a (Huey and Hugli, 1985) and is then recycled to the cell surface (Van Epps *et al*., 1990). Recycling has been proposed to be important for directed cellular migration in a gradient of C5a (Naik *et al*., 1997) but is not apparently related to the clearance of C5a from plasma (Oppermann and Gotze, 1994).

C5a binding to C5aR

The human C5aR binds C5a with a K_d of 1 nM but has an affinity for C5a desArg that is 10 to 100-fold lower.

Table 1 C5aR expression on non-myeloid cell lines

Loci	*Cell Type*	*Reference*
Circulatory system	Mouse microvascular endothelial cells	(Laudes *et al*., 2002)
CNS	Microglia, reactive astrocytes	(Gasque *et al*., 1997)
	Neural stem cells	(Rahpeymai *et al*., 2006)
	Neurons	(O'Barr *et al*., 2001)
	Oligodendrocytes	(Nataf *et al*., 2001)
Connective tissue	Mast cell subtypes	(Oskeritzian *et al*., 2005)
	Synoviocytes	(Yuan *et al*., 2003)
	Human synovial mast cells	(Kiener *et al*., 1998)
	Human articular chondrocytes	(Onuma *et al*., 2002)
Eye	Retinal pigment epithelial cell line	(Fukuoka and Medof, 2001)
Circulatory system	Septic cardiomyocytes	(Niederbichler *et al*., 2006)
Immune system	CD3+ murine T cells	(Connelly *et al*., 2006)
	Human tonsillar B cells	(Ottonello *et al*., 1999)
	Rat thymocytes	(Riedemann *et al*., 2002a)
	Plasmacytoid dendritic cells	(Gutzmer *et al*., 2006)
Kidney	Cultured human renal glomerular mesangial cells	(Braun and Davis, 1998)
	Human renal proximal tubular cells	(Fayyazi *et al*., 2000)
Liver	HepG2 cells	(Buchner *et al*., 1995; Haviland *et al*., 1995; McCoy *et al*., 1995)
	Hepatic stellate Kupfer cells	(Schlaf *et al*., 2003)
	Stimulated hepatocytes	(Koleva *et al*., 2002; Schlaf *et al*., 2003; Schieferdecker *et al*., 2000)
Lung	Human and mouse bronchial epithelial and smooth muscle	(Drouin *et al*., 2001; Floreani *et al*., 1998)
	Rat alveolar epithelia cells	(Riedemann *et al*., 2002b)
Skin	Inflamed keratinocytes	(Fayyazi *et al*., 1999; Zwirner *et al*., 1999)

Ribosomal protein S19 and bacterial chaperone Skp have both been reported to bind to C5aR, although only one laboratory has reported these findings to date (Shrestha *et al*., 2004; Nishiura *et al*., 1996), and the receptor-binding mechanism remains obscure. The potential roles of these non-complement-derived C5aR ligands have recently been reviewed (Yamamoto, 2007). The ligand-binding sites on C5aR have been mapped by a number of methods. Antibodies directed against the N-terminal domain have been shown to inhibit the binding of C5a (Morgan *et al*., 1993; Oppermann *et al*., 1993), and deletion of the N-terminus also prevents C5a binding (Mery and Boulay, 1993; DeMartino *et al*., 1994). A chimeric form of C5aR, with the N-terminus of the receptor for the closely related anaphylatoxin C3a, C3aR, also loses the ability to bind C5a (Crass *et al*., 1999a). However, in all of these cases, peptide analogues of the C-terminus of C5a have still been able to activate the receptor, indicating the presence of an additional binding site.

To identify this site, C5aR chimerae containing the analogous domains of the formyl peptide receptor showed that the second and third extracellular loops (ECLs) of C5aR were essential for ligand binding (Pease *et al*., 1994). A series of powerful genetic studies using a yeast selection system has provided a great deal of evidence for the roles of the ECLs and the TM helices in the formation of the ligand-binding site. These experiments coupled the human C5aR to endogenous G proteins that normally mediate responses to mating pheromones, driving the expression of HIS3 and allowing growth on media lacking histidine (Baranski *et al*., 1999) when C5aR is activated by co-expressed human C5a. Using this system, functional receptors have been selected from large libraries of C5aR molecules that have undergone random saturation mutagenesis. Helix by helix and loop by loop, those residues critical for ligand binding, receptor oligomerization, activation and G-protein coupling have been described (Baranski *et al*., 1999; Geva *et al*., 2000; Gerber *et al*., 2001b; Floyd *et al*., 2003; Klco *et al*., 2003, 2005, 2006; Hagemann *et al*., 2006; Matsumoto *et al*., 2007b). It is not clear how sensitive this system is, and residues that make a small contribution to signal transduction by the ligand may be missed. In addition, the need for C5a to be produced by yeast, rather than being added exogenously because of the impermeability of the cell membrane, may lead to very high concentrations of C5a in the proximity of C5aR, which could further reduce sensitivity. However, despite these minor caveats, this powerful and elegant system has produced many exciting results, some of which are discussed below.

Taken together, the data obtained from all of these experimental approaches have led to the two-site model of receptor activation in which there is a primary high affinity contact between basic residues in the core of C5a (Figure 2) and acidic residues in the N-terminus of C5aR (Figure 3) plus a secondary interaction between the C-terminus of C5a and a binding pocket formed by hydrophobic residues in the TM domains and charged residues at the base of the ECLs. The contributions of these different regions are discussed below.

Binding sites on the C5aR N-terminus

Mapping of the interaction site at the N-terminus of C5aR has been performed in a number of ways, with antibodies targeted to this region and N-terminal deletions having similar inhibitory effects on ligand binding to hC5aR. Identification of the actual residues involved has been problematic, however. A multiple mutant of hC5aR (Asp15,16,18,21Asn) showed a 40-fold decrease in hC5a affinity, and hC5aR(Asp10,15,16,18,21Asn) showed a 133-fold reduction (DeMartino *et al*., 1994). In contrast, the single mutations of Asp10Asn and Asp27Asn or a double mutation (Asp21,27Asn) had no effect on hC5a binding, whereas the multiple substitutions hC5aR(Asp10, 15, 16Asn) or hC5aR(Asp15,16,21,27Asn) showed no detectable hC5a binding (Mery and Boulay, 1994). Similarly, a nuclear magnetic resonance (NMR) study on the hC5aR N-terminus highlighted the importance of residues 21–30 in hC5a binding (Chen *et al*., 1998). O-sulphation of tyrosine residues has been shown to be important for the formation of the ligand-binding site in several GPCR, such as CXCR4 and CCR5 (Hsu *et al*., 2005). In hC5aR, residues Tyr11 and Tyr14 have been shown to be sulphated; the mutation Tyr11Phe showed almost complete loss of C5a binding and Tyr14Phe showed $\sim$50% loss of binding affinity, whereas mutation of Tyr8 had no effect on either sulphation or ligand binding, suggesting that sulphation is essential for the formation of the ligand-binding site on hC5aR (Farzan *et al*., 2001). Providing some support for these findings, a yeast random saturation mutagenesis (RSM) screening study on the N-terminus also found that residues 24–30 were likely to be important for C5a binding (Hagemann *et al*., 2006) but that no single Asp residue was critical. However, this study also found that Tyr11 and Tyr14 could be substituted by a range of other amino acids and so were unlikely to be involved in ligand binding in apparent contradiction of the mutagenesis data. This is probably because yeast lack protein tyrosine O-sulphation machinery (Moore, 2003), and the maintenance of ligand binding in the yeast system may suggest that the high periplasmic concentrations of C5a that occur could be compensating for a low affinity of binding by non-sulphated C5aR.

Binding sites on the C5aR ECLs

Point mutagenesis studies have identified several critical residues in the juxtamembrane regions of these loops, including Arg175, Glu199, Arg206, Asp282. It has been proposed that the receptor interaction site for the C-terminal carboxylate of C5aR agonists is at Arg206, a residue at the extracellular face of helix 5 (Gerber *et al*., 2001a). Mutation of Arg206 to Ala has only a small effect on receptor activation by C5a (Cain *et al*., 2001). Taken together with the observation that C5a des-Arg74 binds to, but does not activate, Arg206Ala-C5aR (Cain *et al*., 2001), it is possible that mutation of this receptor residue perturbs the global structure of the receptor rather than disrupting specific ligand interactions.

This view is further supported by the finding that a ligand-independent constitutively active C5aR mutant (Ile124Asn/Leu127Gln) can be completely deactivated by substitution of Arg206 by His (Gerber *et al*., 2001a). Another potential receptor site for interaction with the C-terminal carboxylate is Arg175, located either on the extracellular face of helix 4 or in the adjacent loop. The analogous residue (Arg161) in the closely related C3a receptor has been proposed to interact with the C-terminal carboxylate of C3a (Sun *et al*., 1999). We have previously shown that although C5aR mutated at Arg175 is only weakly activated by C5a, it can be strongly activated by a mutant form of C5a des-Arg74 isolated from a randomly mutated C5a des-Arg74 library (Cain *et al*., 2003), suggesting that a specific and important interaction between C5aR and C5a is lost when Arg^{175} is mutated to either Ala or Asp. A possible explanation of the data is that the peptide carboxylate makes interactions with both Arg206 and Arg175 at different points in the receptor binding and activation process. Asp282, at the extracellular face of helix 7, has been shown to interact with the side chain of Arg74 of C5a, and with the C-terminal Arg in peptide analogues (Cain *et al*., 2001, 2003). The mutation Glu199Lys has a complete lack of responsiveness to agonists lacking a C-terminal Arg, such as C5a des-Arg74 and C5a[Ala74], suggesting that in addition to a previously demonstrated interaction between Lys68 of C5a and receptor Glu199 (Monk *et al*., 1995; Crass *et al*., 1999b), the side chain of the C-terminal Arg74 residue interacts with Glu199. However, the loss of this interaction following mutation of Glu^{199} has no effect on the responsiveness to C5a, possibly suggesting only a transient interaction between Arg74 and Glu199, with a more important interaction occurring between Arg74 and Asp282. This is clearly shown by the mutation Asp282Arg, which has a very low responsiveness to C5a, but a relatively normal response to C5a des-Arg74 and similar ligands (Cain *et al*., 2001, 2003). Highly conserved Cys residues in loop 1 (Cys109) and loop 2 (Cys188) have been shown to be critical for receptor expression, probably owing to the formation of a stabilizing disulphide linkage (Kolakowski *et al*., 1995).

The yeast RSM screening system described above has confirmed the identification of Arg206 as a key residue, since the only allowed mutation is to Lys. Similarly, the importance of Asp282, where no substitutions were detected, is also confirmed (Baranski *et al*., 1999; Klco *et al*., 2006). Yeast screening of the second ECL, in particular, has provided a plethora of information on this key structure: Arg175 is relatively highly conserved, although only Cys188 was regarded as critical, most probably because of the disulphide linkage that this residue makes with Cys109. However, even more interesting was the finding that several of the mutated receptor sequences were constitutively active, suggesting that EC2 is a negative regulator of receptor activation (Klco *et al*., 2005). Genetic mapping of the first ECL revealed the importance of receptor activation of the Trp-Phe-X-Gly motif that is highly conserved in the GPCR superfamily (Klco *et al*., 2006), although these residues do not contribute to the formation of the ligand-binding site.

Binding sites on the TM helices

Several mutagenesis studies have investigated the role of residues in the TM helices. Asp82 in TM-II has been shown to be critical for signalling but not ligand binding by C5aR (Bubeck *et al*., 1994; Monk *et al*., 1994b; Kolakowski *et al*., 1995). A systematic analysis of Pro and Cys residues in the helices determined several that were critical for ligand binding (Pro170, Cys221) and/or signalling (Pro36, Pro170, Pro214, Cys86, Cys157, Cys285). The yeast RSM screening system enabled the identification of a TM residue, Ile116 in TM-III, as being involved in receptor antagonism. The key role of a binding site in the vicinity of Ile116 was recently confirmed using site-specific disulphide capture, a technique in which potentially interacting amino acids in both ligand and receptor are substituted by Cys residues. The formation of a disulphide linkage indicates that these residues are in close proximity during the binding process. In this way, Leu117, Pro113 and Gly262, residues predicted to be near Ile116 in C5aR models, have been identified as interacting with ligands (Buck *et al*., 2005). The site-specific disulphide capture methodology has also been used to screen a library of thiol-containing small molecules for C5a mimics (Buck and Wells, 2005). Other TM residues identified by the high degree of conservation in yeast RSM screens include Tyr222 and Leu112 (Baranski *et al*., 1999), which are also suggested to be important in receptor function due to conservation in other GPCR. In fact, by assuming that residues with side chains located at helix/helix interfaces are likely to be most highly conserved because of the complementary shapes required to pack helices together, the likely relative orientations of the helices can be mapped (Geva *et al*., 2000). Patches of preserved residues on helices I and II have also suggested a potential interaction site for other membrane proteins or specialized lipids; alternatively, this region could be involved in homodimer formation (Geva *et al*., 2000).

Ligand binding by C5L2

Human C5L2 is a high affinity receptor for C5a that also binds C5a des Arg with a much higher affinity than C5aR (EC_{50} values for C5a and C5a des Arg are 7 and 36 nM, respectively), whereas mouse C5L2 binds mouse C5a des Arg with a 4000-fold higher affinity than mouse C5a (Scola *et al*., 2007). Although C5L2 binding of C5a and C5a des Arg has been confirmed by several groups (Cain and Monk, 2002; Okinaga *et al*., 2003; Johswich *et al*., 2006), the reported ability of C5L2 to bind other anaphylatoxins such as C3a des Arg (Kalant *et al*., 2003) remains a controversial issue. However, a recent paper (Johswich *et al*., 2006) has suggested that the binding of C3a des Arg may have been an artefact of the binding protocol rather than specific binding to C5L2. C5L2 has a similar pattern of tyrosine and acidic N-terminal residues to the C5aR, which have been shown to be a major feature of extracellular binding of C5a (Figure 1b). C5L2 also shares similarities with the C5aR in the number of charged and hydrophobic residues in the loops and TM regions, which are involved in the interaction with the C-terminus of C5a. Despite these common features, ligand binding by the

two receptors is clearly different. Antibodies directed against the N-terminal domain or mutation of tyrosine and acidic residues in the C5L2 N-terminus significantly inhibit C5a des Arg binding but have little effect on the interaction with C5a (Scola *et al*., 2007).

Peptide and peptidomimetic ligands for C5aR

Peptide and peptidomimetic compounds have been developed as small molecule regulators of C5aR. The full agonist activity of C5a is located in the C-terminal 8 residues (Kawai *et al*., 1991) and Abbott researchers derived synthetic peptide analogues as agonists at C5aR that inhibit C5a binding with Ki values of $\sim$300 μM. A decapeptide analogue, Tyr-Ser-Phe-Lys-Pro-Met-Pro-Leu-*D*Ala-Arg, is a full agonist against C5aR at low μM concentrations (Finch *et al*., 1997) that also binds to the C3a receptor, C3aR (Proctor *et al*., 2004). L156,602 (Figure 5) is a cyclic peptide produced by *Streptomyces* with a weak ability to inhibit C5a binding ($IC_{50} = 2\ \mu$M), but toxicity has prevented further development as a C5a antagonist (Tsuji *et al*., 1995). Poly-L-Arg and protamine were expected to inhibit C5a binding owing to the presence of the basic residues from C5a but lack of selectivity has prevented these compounds from being used further (Olsen *et al*., 1988). Structure/activity studies by Abbott researchers on the C5a sequence resulted in a very active hexapeptide agonist, *N*-MethylPhe-Lys-Pro-*D*Cha-Cha-*D*Arg-CO_2H, which has an IC_{50} of $\sim$25 nM against C5aR of isolated polymorphonuclear leukocyte (PMN) membranes (Kawai *et al*., 1992). Substitutions at position 5 by Merck researchers resulted in a partial agonist when cyclohexylalanine (Cha) was replaced with Phe (Drapeau *et al*., 1993) but a full antagonist when replaced by Trp (Konteatis *et al*., 1994), namely *N*-MethylPhe-Lys-Pro-*D*Cha-Trp-*D*Arg-CO_2H (Trp5).

The latter compound was the first pure antagonist with no agonist activity even at 100 μM. It was a potent antagonist (inhibiting binding $IC_{50} \sim 200$ nM), receptor activation by 100 nM C5a ($IC_{50} \sim 85$ nM), on human PMN (Wong *et al*., 1998; Finch *et al*., 1999). In 1995, in the Centre for Drug Design and Development (3D Centre), it was suspected (Fairlie *et al*., 1995) that the Pro-*D*Cha pairing in Trp5 might favour a tight reverse turn motif around Pro as in other macrocycles (Fairlie *et al*., 1995; Chalmers and Marshall, 1995) and that it might be possible to stabilize this through a Lys side chain to C-terminus cyclization. Although Trp5 had no discernible structure in water, 1H NMR spectra subsequently showed in early 1996 that Trp5 had a well-defined gamma turn structure in the dipolar aprotic solvent DMSO (Wong *et al*., 1998), which we had previously used with some success to predict structure of short peptides in membrane environments. Based on our notion that this may be the active conformation, we deployed the unusual side chain–main chain cyclization constraint, resulting in mid-1996 in much more potent and chemically stable antagonists (Wong *et al*., 1998, 1999a; Finch *et al*., 1999; March *et al*., 2004). Among these cyclic antagonists was 3D53, AcPhe[Orn-Pro-DCha-Trp-Arg], cyclized through the side chain of Orn and the terminal carboxylate (Wong *et al*., 1999b), with an IC_{50} of 60 nM for the inhibition of C5a binding to whole PMNs and 30 nM for the inhibition of PMN degranulation (Finch *et al*., 1999). This peptide has been the most intensively evaluated C5a antagonist (Taylor and Fairlie, 2005) with high affinity for dog, cat and rat PMN C5aR ($IC_{50} = 40$ nM) but lower affinity for mouse PMN C5aR ($IC_{50} > 10\ \mu$M) (Woodruff *et al*., 2001) and antagonist activity at cells transfected with human, gerbil (*Meriones unguiculatus*) but not mouse C5aR (Waters *et al*., 2005). It was stable to rat serum, gastric fluid and gastric enzymes (Taylor and Fairlie, 2005).

Of great interest was the demonstration that 3D53 and analogues were potent inhibitors of C5a-induced neutrophil chemotaxis and cytokine production from macrophages *in vitro* (Haynes *et al*., 2000), and these properties were also consistently evident *in vivo*. Interestingly, 3D53 showed little ability to block C5a or C5a des Arg binding to C5L2 (Otto *et al*., 2004), reinforcing suggestions that the two C5aRs have different ligand-binding mechanisms. Although only 1% orally bioavailable or slightly more for analogues without the amide bond connecting the cycle to N-terminal appendages (March *et al*., 2004), a little indiscriminant in binding to GPCRs resulting in off-target side effects (Schnatbaum *et al*., 2006), and expensive to manufacture, 3D53 did show significant efficacy following i.v., p.o., s.c. and t.d. administration in a variety of rat models of inflammatory disease (Table 2) (Kohl, 2006). It was licensed as PMX53 for clinical development by Promics Ltd (subsequently taken over by Peptech Pty Ltd). Other cyclic analogues with more favourable pharmacokinetics, for example JPE-1375 (Schnatbaum *et al*., 2006) and JSM-7717 (http://www.jerini.de/cms/en/02-drug-pipeline/02–02-compounds-and-targets/mater-content-compounds-targets.php), have been developed but results in clinical trials are not yet available.

Non-peptidic ligands for C5aR

To overcome problems associated with peptides, some development of cheaper, orally more bioavailable and more target-selective non-peptidic compounds as either agonists or antagonists has taken place, with at least five groups known to have non-peptidic compounds in development. Early non-peptidic ligands (Figure 4) were of only low-moderate affinity antagonists for human C5aR, such as Merck's aminoquinolines (Lanza *et al*., 1992) and Rhone-Poulenc's phenylguanidines such as RPR121154 ($IC_{50} = 0.8\ \mu$M), which completely inhibited the respiratory burst response of human neutrophils to 100 nM C5a (Astles *et al*., 1997). The basic nature of RPR121154 suggests that it may mimic a positively charged receptor-binding site in the core domain of C5a, although there is no evidence for this mechanism. Merck reported several other structural types of antagonists with submicromolar potencies (De Laszlo *et al*., 1997), but they were not developed further due to partial agonist responses. Interestingly, the hydantoin shown in Figure 4 was a potent full agonist, $EC_{50} = 20$ nM (De Laszlo *et al*., 1997).

The optimization of a series of substituted phenylguanidines led to Mitsubishi Pharma's tetrahydronaphthalene-based compound W54011 (Figure 4), which is a competitive

Table 2 C5aR cyclic peptide antagonist 3D53 and analogues in disease models

Disease	*Animal model*	*Dose/delivery route*	*References*
Arthritis	Rat Monoarticular Antigen-Induced	1–3 mg/kg/day p.o.	(Woodruff *et al.*, 2002)
	Rat Adjuvant-Induced	1 mg/kg/day p.o.	Unpublished
	Rat Collagen-Induced	1 mg/kg/day p.o.	Unpublished
	Rat Paw Oedema	1 mg/kg/day p.o.	Unpublished
Fetal Miscarriage	Mouse Antiphospholipid Abs	50 μg/mouse i.p	(Girardi *et al.*, 2003)
Cardiac Fibrosis	Rat Hypotensive (DOCA)	1 mg/day p.o.	(Mirkovic *et al.*, 2002)
Glomerulonephritis	Rat Antibody-induced	1–10 mg/kg i.v./p.o.	Unpublished
Haemorrhagic Shock	Rat Aorta Aneurysm	1 mg/kg i.v.	(Harkin *et al.*, 2004)
Huntington's Disease	Rat Neuronal Damage	10 mg/kg p.o.	(Woodruff *et al.*, 2006)
Immune Complex Disorder	Rat Arthus	1 mg/kg i.v.	(Short *et al.*, 1999)
	Rat Peritoneal Arthus	1–10 mg/kg p.o.	
	Rat Dermal Arthus	0.4–1 mg t.d., 1–10 mg/kg p.o.	(Strachan *et al.*, 2000, 2001)
Inflammatory BowelDisease	Rat (TNBS-induced)	10 mg/kg p.o., 0.3 mg/kg s.c.	(Woodruff *et al.*, 2003)
Influenza	Mouse	1 mg/kgi.p.	(Kim *et al.*, 2004)
Liver Injury	Mouse	1 mg/kg i.p.	(Strey *et al.*, 2003)
Lung Injury	Mouse	1 mg/kg intratracheally.	(Huber-Lang *et al.*, 2002)
Lupus Nephritis	Mouse SLE	1 mg/kg/day s.c.	(Bao *et al.*, 2005)
Reperfusion Injury	Rat Intestinal	1 mg/kg i.v.; 10 mg/kg p.o.	(Arumugam *et al.*, 2002)
	Mouse Intestinal	25 μg/mouse i.v.	Fleming *et al.*, 2003
	Rat Kidney	1 mg/kgi.v., 10 mg/kg p.o.	(Arumugam *et al.*, 2003)
	Rat Liver	1 mg/kg i.v. 10 mg/kg p.o.	(Arumugam *et al.*, 2004)
	Rat Limb	1 mg/kg i.v., 10 mg/kg p.o.	(Woodruff *et al.*, 2004)
Sepsis	Rat Neutropaenia (C5a, LPS, cobra venom factor)	0.3–3 mg/kg i.v.	(Saatvedt *et al.*, 1996; Short *et al.*, 1999; Taylor and Fairlie, 2005)
		10 mg/kg p.o.	(Strachan *et al.*, 2001)
	Mouse Caecal Ligation	50 mg/kg topical	Unpublished
		1–3 mg/kgi.v., 10 mg/kg p.o.	(Huber-Lang *et al.*, 2002)

Abbreviations: DOCA: deoxycorticosterone acetate; TNBS: trinitrobenzene sulphonic acid; SLE: systemic lupus erythematosus; LPS: lipopolysaccharide.

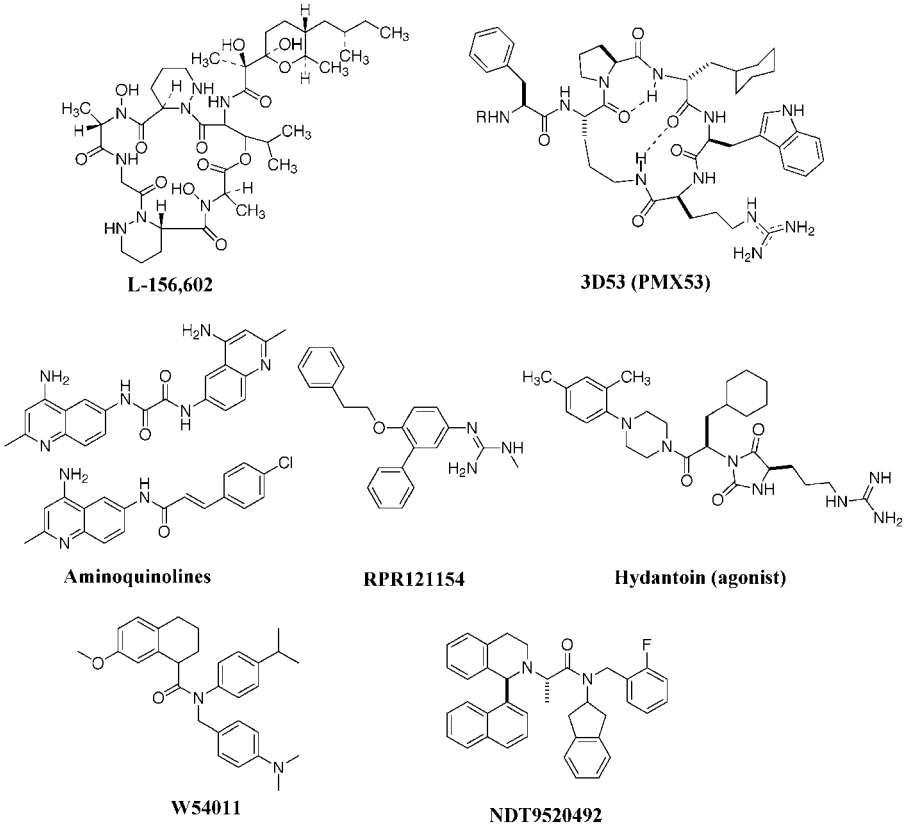

Figure 4 Structures of small molecule ligands for C5aR.

non-peptidic C5aR antagonist ([125I]hC5a $IC_{50} = 2.2$ nM) that inhibits intracellular Ca^{2+} mobilization, chemotaxis and production of reactive oxygen species with $IC_{50} = 3.1$, 2.7 and 1.6 nM, respectively (Sumichika *et al*., 2002). The combination of potency and oral availability appeared promising but its substantial hydrophobicity and problems with species specificity (active for human, cynomolgus monkey and gerbil but not mouse, rat, guinea pig, rabbit or dog neutrophils) complicated pre-clinical studies.

NDT9520492 (Figure 4) is a member of a large series of compounds developed by Neurogen Corp with C5aR antagonist activity (Waters *et al*., 2005) at human and gerbil but not mouse C5aR. A similar compound, NGD 2000–1 had no therapeutic effect in an asthma study and, while a Phase II trial in RA showed some promise, the compound inhibited cytochrome P450 3A4 and development was halted (http://www.neurogen.com/products/c5aindex.htm). Among other non-peptidic C5a antagonists is Jerini JSM7431, which appears to have been discontinued.

Binding of small molecule ligands to C5aR

Unlike the binding of proteins to C5aR, small molecule ligands bind primarily in the TM region of the receptor. Recently, the putative binding site on C5aR has been reported for the linear antagonist Trp5 (Gerber *et al*., 2001b; Higginbottom *et al*., 2005) and the cyclic antagonist 3D53 (Higginbottom *et al*., 2005), based on a combination of studies that included molecular modelling of the receptor, molecular docking of NMR structures of the ligands into the homology model of the receptor, site directed mutagenesis of the receptor and structure-activity studies for various ligands binding to wild type versus mutant C5aR on PMNs. Mapping of ligand binding using these methods suggested that Trp5 and 3D53 bind at the same (or slightly overlapping) location in the TM region of C5aR near the extracellular interface (Figure 5). Key receptor residues were thought to be on TM-III: Ile116 (3.32), Tyr121 (3.37), TM-V: Glu199, (5.35), Arg206 (5.42), Phe211 (5.47), Leu215 (5.51)), TM-VI: Phe251 (6.44), Trp255 (6.48) and TM-VII: Asp282 (7.35), Val286 (7.39), Tyr290 (7.43); the numbers in brackets show residue positions according to Ballesteros and Palczewski (2001) The model reflects the importance within 3D53 of Arg, D-Cha and Ac-Phe components as binding residues and the Trp as an antagonist-determining residue. It also places the Ac-Phe appendage on the cycle in the vicinity of extracellular loop two (not shown), which is thought to act as a lid on the ligand-binding active site. Flexible peptide agonists reversibly enter the hydrophobic 'pit' in the TM region of the receptor, but Trp5 and especially 3D53 occupy the cavity may hold the ECL2 lid down. This may be the reason why it is difficult to dissociate 3D53 from the receptor (slow off rate) and why it has an insurmountable mechanism of antagonism.

Non-peptidic ligands such as W-54011 (Sumichika *et al*., 2002) and NDT9520492 (Waters *et al*., 2005) appear to bind in very similar locations within the TM region of C5aR as the Trp and D-Cha side chains of 3D53, based at this time on scant evidence from effects of species dependence in neutrophil C5aR or site-directed mutation of C5aR residues (for example Trp213 (5.49)). Thus, W-54011 potently inhibits C5a-induced intracellular Ca^{2+} mobilization in neutrophils of cynomolgus monkeys and gerbils but not mice, rats, guinea pigs, rabbits and dogs. It is important to point out that the IC_{50} values reported for competitive reversible antagonists W54011 and NDT952492 are biased by the low nM concentrations of C5a that they were measured against. Neither non-peptidic compound is as effective as the insurmountable cyclic peptide antagonist 3D53 at higher C5a concentrations, a distinction that we attribute to the unique ability of 3D53 to fill the hydrophobic C5aR cleft and close the ECL2 loop lid on the cleft. The smaller non-peptidic compounds reported to date simply do not occupy enough space to interact strongly with the lid of the cavity while being anchored in their binding sites and are readily displaced.

Intracellular signalling via C5aR

C5aR primarily couples to Gαi2 (Sheth *et al*., 1991; Skokowa *et al*., 2005), a pertussis toxin (PT)-sensitive G protein. However, ectopically expressed C5aR, and also C5aR in some

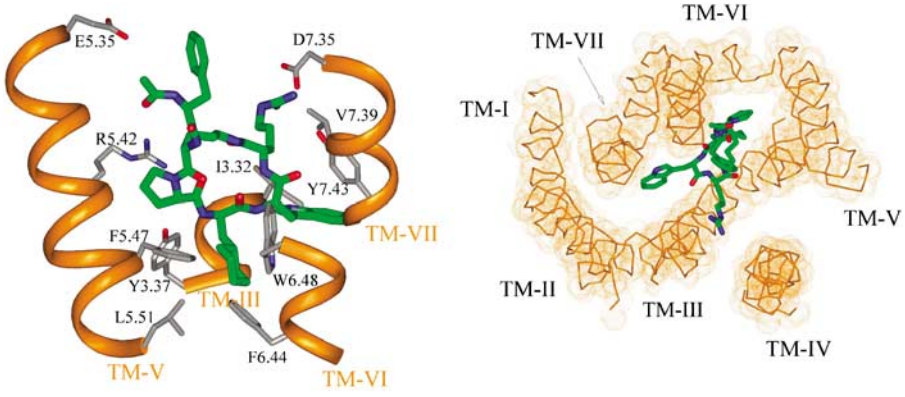

Figure 5 Modelled interaction between 3D53 (green) and human C5aR (orange) showing ligand-binding pocket (left: side view, right: top view) with Arg, Trp, dCha and AcPhe components of 3D53 fitting between helices of C5aR with key receptor residues labelled according to Ballesteros and Palczewski (2001). Figure updated since Higginbottom *et al*. (2005).

haemopoietic cell types such as monocytes, can also couple to Gα16 (Monk and Partridge, 1993; Buhl *et al*., 1994; Kalant *et al*., 2003), a PT-insensitive G protein. The loss of Gβ1 and Gβ2 (effectively no Gβ expression at all) in J774 mouse macrophages eliminates all responsiveness to C5a (Hwang *et al*., 2005), whereas the loss of Gβ1 alone does not affect chemotaxis (Hwang *et al*., 2004). Recently, C5aR has been shown to be able to couple to a wide range of G proteins when key intracellular residues are mutated, suggesting that the regulation of the G-protein coupling range occurs by a mechanism of repression rather than by positive promotion of interactions (Matsumoto *et al*., 2007b). C5aR also couples directly or indirectly to a small range of other intracellular proteins (Figure 6). The Wiskot–Aldrich syndrome protein (WASP) was detected as a binding partner of the C-terminus of C5aR using a yeast 2 hybrid assay (Tardif *et al*., 2003). WASP binding was strongly potentiated in the presence of active cdc42, a small guanine-5′-triphosphate (GTP)-binding protein, suggesting that the association occurs after C5aR activation. WASP is a multifunctional protein with a role in the regulation of actin dynamics (Ochs and Notarangelo, 2005), and so could be involved in the chemotactic response to C5a.

Activated C5aR has also been shown to associate with two of the four mammalian β-arrestins (β-arrestin 1, 2), which have different dependencies on the phosphorylation status of the receptor (Braun *et al*., 2003). The arrestins have multiple roles, being involved in receptor trafficking and the regulation of signalling (Gurevich and Gurevich, 2006). G-protein receptor kinases (GRK) are thought to control the phosphorylation levels of C5aR and most likely GRK2 and GRK3, which are found co-expressed with C5aR in cell lines such as HMC-1 (Langkabel *et al*., 1999). However, overexpression of GRK2 (and 6) failed to alter phosphorylation patterns of C5aR (Milcent *et al*., 1999) and so it is possible that only GRK3 is involved in C5aR phosphorylation *in vivo*. Apart from their role in phosphorylating GPCR, GRK also interact with a range of other signalling molecules, including Akt, MAPK/ERK kinase (MEK) and phosphatidylinositol 3-kinase (PI3K), suggesting a wider role in connecting GPCR with diverse signalling pathways (Ribas *et al*., 2007). RGS1, an effective GTPase-activating protein (GAP) for G subunits of the Gi and the Gq family has been shown to be involved in C5aR desensitization (Denecke *et al*., 1999). C5aR has been reported to activate phospholipase C (PLC)β2 but not PLCβ3 in a PT-sensitive manner (Jiang *et al*., 1996) in Cos7 cells, although the ability of transfected human C5aR to stimulate PLC activity in rat basophilic leukaemia cells, which express only PLCβ3 (Ali *et al*., 1997), suggests that C5aR may also couple to this isoform.

In neutrophils, C5a leads to causes downstream activation of p21-activated kinases (PAK), which are downstream effectors of cdc42 and rac GTPases (Huang *et al*., 1998) as well as G protein γ subunits; PAK family members are involved in altering cell morphology/chemotaxis, the activation or potentiation of several distinct MAPK cascades and the activation of nuclear factor-κB (NF-κB) in macrophages (Bokoch, 2003). Interestingly, the PAK-associated guanine nucleotide exchange factor (PIXα) also binds to PAK1 and, in association with G$\beta\gamma$ subunits, forms a complex that can activate cdc42 (Li *et al*., 2003) in a positive feedback loop. GIT2, a GAP that regulates Arf activity, also associates with PAK and is indispensable for direction sensing in C5a-stimulated neutrophils (Mazaki *et al*., 2006); GIT2 is further involved in controlling the production of superoxide anions during chemotaxis and in orienting superoxide production towards the source of chemoattractant (Mazaki *et al*., 2006).

C5a can activate the transcription factor, cAMP response element-binding protein (CREB), by phosphorylation at the convergence of two pathways, PI3K/Akt and extracellular signal-regulated kinase (ERK) signalling (Perianayagam *et al*., 2006); CREB activation has been proposed to be a part of the mechanism by which C5a can delay neutrophil apoptosis (Perianayagam *et al*., 2002, 2004) and prolong an inflammatory response. p38a MAPK is activated by PAK1/PAK2 and, in turn, activates MAPK-activated protein kinase 2 (MAPKAP-K2); thus, in primary macrophages from MAPKAP-K2 deficient mice, chemotaxis to C5a is impaired (Rousseau *et al*., 2006); heat-shock protein HSP-27 is a likely substrate of MAPKAP-K2 in these cells. The p38 MAPK inhibitor, SB203580, can inhibit C5a-induced migration in a mouse acute lung injury model (Nash and Heuertz, 2005). RhoG in murine neutrophils may be involved in Rac1 and Rac2 activation, leading to nicotinamide adenine dinucleotide phosphate oxidase activation (Condliffe *et al*., 2006). C5a activates the PI3K/Akt signalling pathway and induces the phosphorylation of the p38a MAPK, ERK and c-Jun N-terminal kinase, leading to suppression of IL-12 production in human monocytes (la Sala *et al*., 2005) and mouse macrophages (Hawlisch *et al*., 2005).

In human erythroleukaemia cells, signal transducers and activators of transcription (STAT3) phosphorylation can be stimulated by C5a in a PTX-insensitive manner, most likely through Gα16 and the Ras/Raf/MEK/ERK and c-Src/JAK pathways (Lo *et al*., 2003); in contrast, STAT3 phosphorylation occurs only through an ERK pathway in C5a-stimulated neutrophils (Kuroki and O'Flaherty, 1999). In endothelial cells but not leukocytes, C5a-induced motility can be blocked by inhibitors of the epidermal growth factor (EGF) receptor (EGFR) and by neutralizing antibodies against the EGFR and heparin-binding EGF-like factor (Schraufstatter *et al*., 2002); transactivation of EGFR by several GPCRs has been reported and is thought to lead to the amplification of responses.

C5aR can form homodimers (Geva *et al*., 2000; Floyd *et al*., 2003; Klco *et al*., 2003), probably by associations between helices I and II from the partner receptors, and can also complex with other GPCRs in heterooligomers, for instance with CCR5 (Huttenrauch *et al*., 2005). The consequences of these interactions for C5aR are, as yet, unclear; oligomers form early in the biosynthetic pathway and are known to be important during transport to the plasma membrane (Milligan *et al*., 2003) and studies on oxytocin and vasopressin receptors reveal a complex pharmacology after oligomerization (Albizu *et al*., 2006). Ligand binding by C5aR in homo- or hetero-oligomers has not yet been investigated but CCR5 co-expressed with C5aR was found to be phosphorylated after C5a addition, suggesting a role in

the control of other chemoattractants (Huttenrauch *et al*., 2005).

Intracellular protein binding sites on C5aR

The sites on C5aR that control localization and signalling have been investigated in a small number of studies using point mutagenesis of intracellular regions of C5aR. The mutation of Arg68 in intracellular loop 1 or Trp230, Thr235, Thr238 in loop 3 diminishes the ability of C5aR to signal in Cos7 cells that co-express Gα16 (Kolakowski *et al*., 1995). Deletion of the C-terminal 23 amino acids of C5aR had little effect on Gαi protein-dependent signalling in transfected mammalian cells (Monk *et al*., 1994a), although internalization was dependent on residues 335–350, as shown by a series of C-terminal truncations (Bock *et al*., 1997). This inhibitory effect on internalization is probably due to a loss of phosphorylation sites in the C-terminal domain, as the simultaneous mutation of Ser332, 334 and 338 to Ala also caused an 80% reduction of phosphate incorporation into C5aR (Giannini *et al*., 1995). This reduction leads to a significant retardation of ligand-induced internalization (Naik *et al*., 1997), most likely by loss of an association with other components of the internalization machinery, β-arrestin, clathrin and dynamin (Braun *et al*., 2003). Phosphorylation of Ser334 was found to be critical for the sequential phosphorylation of the other Ser residues in this triplet; mutation of Ser334 to Asp allowed phosphorylation to occur as normal at Ser332, 338 (Christophe *et al*., 2000), followed by the remaining C-terminal Ser residues, 314, 317 and 327. Interestingly, the same authors found that loss of phosphorylation at Ser332 and 334 prevented receptor desensitization even when internalization was still normal, resulting in prolonged responses to C5a. The regions of C5aR involved in coupling to G proteins have been broadly defined using peptide analogues of the intracellular loops and the C-terminus fused to a cell permeant sequence derived from Kaposi fibroblast growth factor (Auger *et al*., 2004). This work showed the proximal region of the C-terminus to be a major G-protein-binding site, with loop 3 having a role in G-protein activation. The intracellular regions of C5aR have also been analysed using the yeast screening method. These studies have given the most profound insight into the coupling between C5aR and G proteins, providing information on how G-protein specificity occurs and on the mechanism of G-protein activation. The C-terminus of C5aR was shown to be dispensable for G-protein coupling, and there were a minority of preserved residues in the first (2/16 residues studied were completely resistant to substitution) and second (6/17 resistant residues) intracellular loops. In contrast, the third intracellular loop was quite highly conserved (11/21 residues) (Matsumoto *et al*., 2007a). Interestingly, an analysis of mutants selected on the basis of efficient coupling to Gαi in yeast showed that many mutations, particularly in the C-terminus and second intracellular loop broaden G-protein specificity and mutants that couple well to Gαq and Gαs-like G proteins were characterized. It was concluded that the normal sequence of C5aR contains negative regulators of specificity that can be disrupted by mutation (Matsumoto *et al*., 2007b).

Signalling via C5L2

Although C5L2 has the conventional structure of a GPCR, studies have found that C5L2 does not couple to G proteins. This is thought to be owing to the lack of a highly conserved Asp-Arg-Tyr motif, found in the third TM domain, which in C5L2 is replaced with delocalized lipophilic cation. In the presence of C5a, cells transfected with C5L2 show no increase in cytosolic calcium levels or activation of the MAP kinase pathway, and C5L2 transfected RBL cells failed to degranulate upon stimulation with C5a or C5a des Arg (Cain and Monk, 2002; Okinaga *et al*., 2003; Johswich *et al*., 2006). When the Asp-Leu-Cys motif of C5L2 is mutated to Asp-Arg-Cys, the binding of C5a can induce a small increase in intracellular calcium levels, suggesting an incomplete restoration of G-protein coupling (Okinaga *et al*., 2003). Other intracellular and TM sequences that are not conserved between C5L2 and C5aR, for instance the Asn-Pro-X-X-Tyr motif in TM-VII and a deleted polar tripeptide in intracellular loop 3 (Figure 3), may also contribute to the inability of C5L2 to couple to signalling pathways. The ability of C5L2 to bind anaphylatoxins without signalling has led to the suggestion that C5L2 may have a role as an anaphylatoxin decoy receptor, thereby regulating the availability of C5a and C5a des Arg. Rat neutrophils stimulated with C5a and LPS, in the presence of a C5L2 blocking antibody, produce dramatically increased levels of IL-6 compared to controls (Gao *et al*., 2005). C5L2-deficient mice produce neutrophils with an increased response to both C5a and C5a des Arg and show a 2- to 3-fold increased influx of neutrophils into the lung of $-/-$ C5L2 animals and higher levels of TNF-α and IL-6 when compared to wt-mice in a model of pulmonary IC injury (Gerard *et al*., 2005). A comprehensive study of sepsis patients found higher C5L2 content in PMN obtained from patients who survived the observation period compared to patients who failed to survive; low C5L2 expression seemed to correlate with sepsis induced multi-organ failure, suggesting an important role for C5L2 in sepsis (Huber-Lang *et al*., 2005). In contrast to these data, a recent report has suggested that C5L2 is a positive modulator for signalling through C5aR (Chen *et al*., 2007). Neutrophils from C5L2-deficient mice responded less strongly to C5a compared to cells from wild-type animals, and reduced numbers of peritoneal macrophages were elicited by thioglycollate. Airway hyper-responsiveness and inflammatory cell infiltration was reduced in C5L2-deficient mice, although these mice were more susceptible to the lethal effects of LPS.

Several studies found that cell lines transfected with C5L2 do not show net loss of receptor from the membrane after ligand binding, suggesting that C5L2 does not undergo ligand-induced internalization (Cain and Monk, 2002; Okinaga *et al*., 2003). The difference in results may be owing to the short time of exposure of C5L2 to ligand used in these *in vitro* studies (5–15 min) compared to patients with sepsis where C5L2 expressing cells would be exposed to

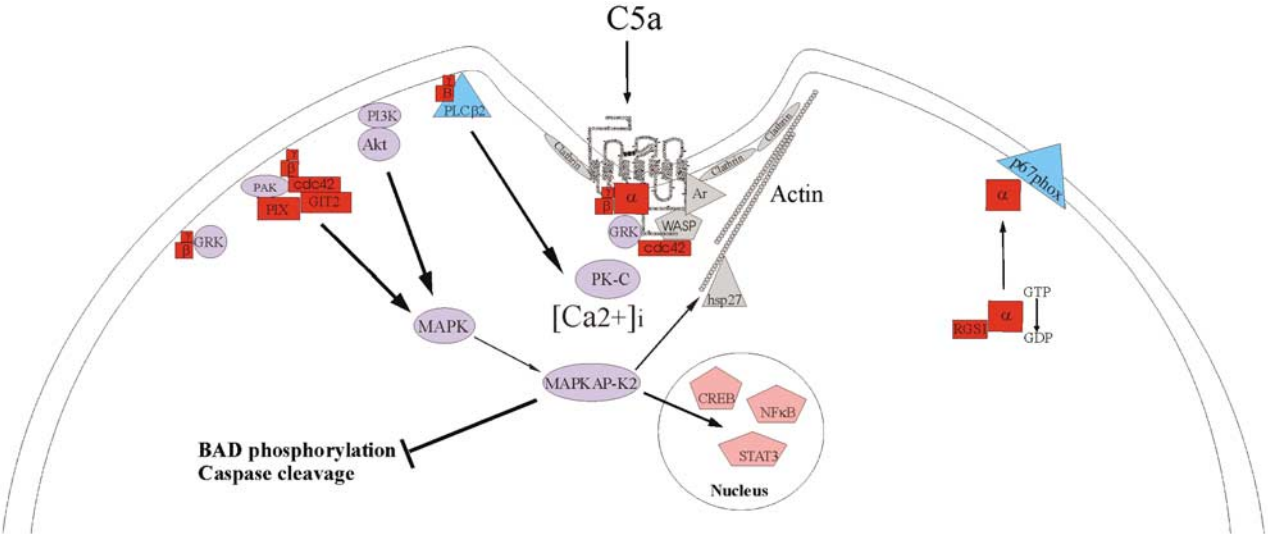

Figure 6 The 'interactome' of C5aR. C5aR interacts directly or indirectly with kinases (purple), GTP binding/regulatory proteins (red), transcription factors (pink), other signalling enzymes (blue) or structural proteins (grey). Internalization of C5aR is mediated by clathrin, which associates with receptor-bound β-arrestin (Ar) and the actin cytoskeleton. Proteins, such as hsp27, phosphorylated by MAP kinase-activated protein kinase 2 (MAPKAP-K2), may regulate the actin cytoskeleton. MAPKAP-K2 is itself activated by the mitogen-activated kinase (MAPK/ERK/JNK) cascade, in turn activated by kinase Akt (also known as PK-B) or by p21-associated protein kinase (PAK) complexed with Rac/Cdc42 guanine nucleotide exchange factor PIXα, cdc42 and G-protein-coupled receptor kinase-interactor 2 (GIT2). G-protein α-subunits are deactivated by regulator of G-protein signalling 1 (RGS1) that stimulates GTP conversion to GDP; in the GDP-bound state, α-subunits can bind to and modulate the activity of the NADPH-oxidase component $p67^{phox}$. $\beta\gamma$-subunits directly activate PAK and indirectly activate PK-Cβ by increasing diacylglycerol and intracellular Ca^{2+} ($[Ca^{2+}]_i$) through phospholipase Cβ (PLCβ). $\beta\gamma$ may be sequestered by G-protein-coupled receptor kinase (GRK), which also phosphorylates C5aR along with PK-Cβ. Transcription factors signal transducer and activator of transcription 3 (STAT3), cAMP responsive element binding protein (CREB) and nuclear factor (NF)-κB are activated at the convergence of the kinase pathways, and apoptosis inhibited by phosphorylation of Bcl-associated death promoter (BAD) and upregulation of caspase degradation. JNK, c-Jun N-terminal kinase; NADPH, nicotinamide adenine dinucleotide phosphate.

anaphylatoxins on a much longer (hours/days) time scale. Therefore, the regulation of C5L2 expression seems to be variable, depending on both cell type and level of exposure to anaphylatoxins, and the mechanisms involved in regulating C5L2 expression have yet to be elucidated. Although C5L2 does not appear to signal using the traditional mechanisms employed by GPCRs, several studies suggest that C5L2 has the ability to induce cellular effects. A recent study (Gavrilyuk *et al*., 2005) found that noradrenaline could upregulate C5L2 message and protein in rat astrocytes, and this correlated with an anti-inflammatory response induced by noradrenaline; transfection of astrocytes by C5L2 down regulated NF-κB activity, whereas antisense oligonucleotides against C5L2 caused the reverse effect. This observation suggests that the presence of C5L2 may exert some inhibitory effects within the cell, although the mechanisms behind such responses are currently unknown. The suggestions that C5L2 can both prevent ligand from interacting with C5aR and downregulate pro-inflammatory signalling raise the possibility that treatments that increase C5L2 expression could be used as part of an anti-inflammatory strategy.

Conclusions

There is now strong evidence of a pathogenic role for C5a from studies in numerous disease models using antibodies to C5a or C5aR, soluble receptor sCR1 and C5aR-knockout and knockin transgenic mice (Weisman *et al*., 1990; Bozic *et al*., 1996; Goodfellow *et al*., 1997; Hopken *et al*., 1997; Mohr *et al*., 1998; Lee *et al*., 2006), and especially from studies of small molecule antagonists (for example Table 2). Alexion now has Phase III data for a C5 antibody (eculizumab) to treat haemolytic anaemia; Avant has Phase IIb data for sCR1 in cardiac bypass surgery (Ratner, 2006); Promics has Phase IIa data for 3D53 in rheumatoid arthritis. However, as therapeutics for chronic inflammatory diseases, such biologics are compromised by high cost, low bioavailability, metabolic instability and the need for repeated injections. The development of effective small molecule antagonists for C5aR is an attractive alternative, and compounds generated to date have accelerated our understanding of the central involvement of C5a in many inflammatory disease states, albeit so far mainly through the use of rodent models of disease. Those studies have demonstrated profound immunoregulatory effects for C5aR antagonists *in vivo* and encouraging benefits in animal models of human inflammatory diseases. One caveat concerning the use of C5aR antagonists should be made, however. The recent demonstration of a protective role for C5aR in the sensitization phase of asthma (reviewed by Kohl and Wills-Karp, 2007) suggests that, although the use of powerful C5aR antagonists may be beneficial for existing inflammatory conditions, patients may become more easily sensitized to new pulmonary allergens. The discovery of C5L2 as an inhibitory C5a/C5a des Arg receptor has also raised the intriguing possibility of the use of this receptor as a novel anti-inflammatory

strategy, although further work is required to determine the full functionality of this protein.

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Conflict of interest

PNM has previously acted as consultant to Jerini AG (2005) and sat on the Scientific Board of Promics Pty Ltd (2005–6). DPF was the Scientific Director, CSO, and a founder of Promics Pty Ltd.

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