The substantial variations in the responses of cells to the anaphylatoxin C5a and its desarginated form, C5adR74, suggest that more than one type of cell surface receptor for these ligands might exist. However, only a single receptor for C5a and C5adR74, CD88, has been characterized to date. Here we report that the orphan receptor C5L2/gpr77, which shares 35% amino acid identity with CD88, binds C5a with high affinity but has a 10-fold higher affinity for C5adR74 than CD88. C5L2 also has a moderate affinity for anaphylatoxin C3a, but cross-competition studies suggest that C3a binds to a distinct site from C5a. C4a was able to displace C3a, suggesting that C5L2, like the C3a receptor, may have a low binding affinity for this anaphylatoxin. Unlike CD88 and C3a receptor, C5L2 transfected into RBL-2H3 cells does not support degranulation or increases in intracellular [Ca2+] and is not rapidly internalized in response to ligand binding. However, ligation of C5L2 by anaphylatoxin did potentiate the degranulation response to cross-linkage of the high affinity IgE receptor by a pertussis toxin-sensitive mechanism. These results suggest that C5L2 is an anaphylatoxin-binding protein with unique ligand binding and signaling properties.

Complement fragment C5a is a potent chemoattractant and anaphylatoxin that acts on all classes of leukocytes and on many other cell types including endothelial, smooth muscle, kidney, liver, and neuronal cells (1, 2). In addition to its proinflammatory effects, C5a has recently been shown to protect cells against toxic insult and to stimulate proliferation in neurons and hepatocytes (3–6), suggesting a wider role for C5a in homeostasis. C5a is rapidly desarginated by serum carboxypeptidase N to the less potent derivative C5adR74, the first stage in deactivation of anaphylatoxin activity (7). The dr74 form has a different spectrum of bioactivity to intact C5a; for instance, in human basophils, stimulation by intact C5a causes the release of lipid mediators (e.g. leukotriene C4) and cytokines (e.g. interleukin-4 and interleukin-13), whereas C5adR74 stimulates only cytokine release (8). Antagonists can also discriminate between different cell types: a cyclic peptide is 30-fold more potent on human neutrophils than a linear peptide antagonist, but both peptides are equally potent on human umbilical artery macrophages (9). Wide variations in antagonist affinity have also been observed in different species, but the sequences of C5a receptor homologs in these species do not suggest an obvious mechanism for these variations (10).

The molecular basis for the ability of different cell types to discriminate between agonists, antagonists, and intact C5a/C5adR74 has yet to be elucidated as only a single receptor for C5a (CD88), a member of the G protein-coupled receptor superfamily, has so far been cloned (11, 12). CD88 is in a G protein-coupled receptor subfamily that contains the receptors for human C3a (C3aR), formyl peptide, and an orphan receptor, C5L2 (also known as gafr77) (13, 14). C5L2 transcripts are widespread with expression demonstrated in spleen, testis, brain, heart, lung, liver, kidney, ovary, and colon and in granulocytes and dendritic cells but not monocyte-derived macrophages (13, 14). Here we report that C5L2 has high affinity binding sites for both C5a and C5adR74, apparently with a distinct binding site for the related anaphylatoxin C3a. Unlike CD88, C5L2 couples poorly to G-like G protein-mediated signaling pathways and does not undergo rapid receptor internalization in response to ligand binding.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—RBL-2H3 cells were routinely cultured in Dulbecco’s modified Eagle’s medium plus 10% (v/v) fetal calf serum, which was supplemented with 400 mg/liter G-418 for transfected cells, at 37 °C in 5% CO2.

**Cloning of C5L2 and C4a and Transfection of RBL Cells**—The C5L2 cDNA was cloned from a human brain (whole) Marathon-Ready cDNA (CLONTECH) by PCR using the sense primer 5’-GCGGCGAACTGTG-CACCATGTACCATAGCAGCTCCAGACTACGCTGGGAAA-GATGTGTGCTACCTAC-3’ and the antisense primer 5’-GGGCCCAGAT-TCTCACCTCCATCTCCGAGAC-3’. The added HindIII and EcoRI restriction sites are shown respectively in italics, the Kozak sequence used is shown underlined, and the added human influenza hemagglutinin (HA) tag on the sense primer is shown in bold. After authentication by sequencing, the full-length PCR product was digested with EcoRI and HindIII (Roche Molecular Biochemicals) and ligated into the expression vector pEE8hCMV.neo (Celltech). The C3aR cDNA, a generous gift of P. Gasque (Cardiff, United Kingdom), was inserted into PE8hCMV.neo vector at the same site. Stable transfection of RBL-2H3 cells was achieved by electroporation as previously described (15). An anti-HA tag monoclonal antibody (clone 12CA5, Roche Molecular Biochemicals) or C3aR (clone P4B4, a generous gift from P. Gasque) was used to sort the highest 5% of transfected cells on a Becton-Dickinson Vantage flow cytometer in three rounds of cell sorting. C4a was cloned from the same human brain library as C5L2 using the sense primer 5’-GCGGCGGATCCTACACGTAAATCCAAAGGGCGA-3’ and the antisense primer 5’-GCACCTGTACCTATATTCGTTGAGGCGCCCT-3’; the added BamHI and KpnI restriction sites are shown respectively in italics.

**Production of Anaphylatoxins**—Expression and purification of the recombinant Hisa-tagged C5a, C5adR74, C3a, and C4a was performed under denaturing conditions as described previously (9). C4a was also expressed and purified under non-denaturing conditions by sonication in the presence of BugBuster Protein Extraction Reagent (Novagen) using

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‡ The abbreviations used are: dR74, des-Arg74; CD88, human C5a receptor; C3aR, human C3a receptor; RBL, rat basophilic leukemia; Fluo3AM, acetoxymethyl ester of Fluo3; HA, hemagglutinin; HSA-DNP, 2,4-dinitrophenol linked to human serum albumin; IgE(DNP) im-munoglobulin E specific for 2,4-dinitrophenol.
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**FIG. 1. Sequence alignment of C5L2** (gp97) with CD88 and C3aR. Transmembrane domains are underlined. Residues involved in receptor activation by ligand in CD88 (Swiss-Prot accession no. P21730) and conserved in C5L2 (gp97) (accession no. Q9P296) and the C3aR (accession no. Q16581) are shown boxed by solid lines; the "DRY" motif residues are marked by *, and the intracellular loop 3 Ser/Thr-containing motif is marked by +.

**RESULTS AND DISCUSSION**

**C5L2 and CD88 Have Very Similar Binding and Activation-related Sequences**—A sequence alignment between CD88, C3aR, and C5L2 is shown in Fig. 1. The N termini of CD88 and C5L2 contain several acidic residues that are characteristic of complement fragment receptors and that, for CD88 at least, form a part of the ligand binding site (19). Both CD88 and C3aR have been shown to have distinct ligand binding and activation sites (20). Receptor activation by ligand involves the engagement of charged and uncharged residues on the extracellular faces of the transmembrane helices. For CD88, these include Ile<sup>116</sup>, Val<sup>206</sup>, Arg<sup>175</sup>, Glu<sup>199</sup>, Arg<sup>206</sup>, and Asp<sup>230</sup>(21–23); C3aR has conserved residues at most analogous positions (Fig. 1). Interestingly C5L2 shares all of these residues except for Asp<sup>230</sup>, where there is a Glu residue (boxed residues in Fig. 1). Thus C5L2 has a similar acidic ligand-binding N-terminal domain to CD88 and a similar ligand activation domain.

**C5L2 Binds Multiple Complement Fragments**—C5L2 was cloned from a human brain cDNA library and expressed as a stable transfectant in the rat basophilic leukemia line RBL-2H3. Antibodies against a N-terminal HA peptide, inserted after the initiating methionine of C5L2, was used to select the top 5% of expressing cells by fluorescence-activated cell sorting in three rounds of cell sorting. Anti-HA antibody, but not anti-CD88 or anti-C3aR monoclonal antibody, recognized these cells (data not shown). Binding assays were performed using <sup>125</sup>I-C5a and <sup>125</sup>I-C3a to determine the ligand specificity. The specific binding curve indicates that specific, saturable binding of C5a occurs (Fig. 2a) with a receptor number calculated from the <i>B<sub>max</sub></i> value of 39,736 ± 5,993/cell, mean ± S.E., <i>n</i> = 3. C3a also binds specifically (Fig. 2b) with a similar number of binding sites (25,652 ± 10,237/cell, mean ± S.E., <i>n</i> = 3; not significantly different from the C5a binding site number), but the calculated affinity for C5a was higher than that for C3a. Ligand specificity was investigated further using competition binding analysis, preincubating cells with a number of poten-
affinity of C5L2 for C5adR might explain the sensitivity of

The ligand binding data suggested the possibility that the high

C5a and C5adR (EC50 than C5a or C5adR. These data demonstrate that C5L2 has a

response to HSA-DNP (Fig. 4). Untransfected cells did not

activity of the recombinant C4a used here may be due to the

production process, which did not include the denaturation/

refolding step used for C5a and C3a. Denaturation of C4a in

extracellular loop that appears to form the binding site for C3a

however, unclear as C5L2 does not have the very large second

arginine of C5L2 was also assessed as the increase in intracellular Ca²⁺

C3aR has been previously reported (25). The signaling activity of C5L2

couples weakly to intracellular signaling pathways—

The ligand binding data suggested the possibility that the high

affinity of C5L2 for C5adR might explain the sensitivity of some cell types to this ligand. We examined this by assessing the ability of C5L2 to activate transfected RBL cells using the degranulation response to ligand binding. The C5L2 ligands (C5a, C4a, C3a, and C5adR) were not able to support degranulation at concentrations of up to 3 μM (Fig. 3a). In contrast, C5a and C5adR (EC50 = 8 and 21 nM, respectively) could activate CD88-transfected RBL cells (Fig. 3b), and C3a (EC50 = 52 nM) but not C4a could activate C3aR-transfected RBL cells (Fig. 3c). C5L2 also did not increase degranulation even when RBL cells were primed with phorbol 12-myristate 13-acetate (100 nM), a treatment that enhances the response to suboptimal stimuli in RBL cells (26), for 10 min prior to the addition of ligand (data not shown) (17). The failure of C4a to activate C3aR has been previously reported (25). The signaling activity of C5L2 was also assessed as the increase in intracellular Ca²⁺ using Fluo3AM-labeled RBL cells, an assay previously shown to be 10-fold more sensitive to ligand concentration than degranulation (15). Cells expressing C5L2 did not respond to 100 nM C5a, C5adR, C3a, or C4a (Fig. 3d), whereas RBL cells expressing CD88 responded to C5a and C5adR with robust increases in cellular fluorescence (Fig. 3e), and RBL cells expressing C3aR responded to C3a but not to C4a (Fig. 3f). Identical patterns of activity were observed using 1 μM ligand (data not shown). The absence of intracellular Ca²⁺ signaling is not due to low receptor number because the receptor expression level for C5L2 (~40,000/cell) is actually higher than CD88 expression (~36,000/cell (17)). We then examined whether ligand binding to C5L2 could prime RBL cells for a subsequent stimulus through the tyrosine kinase-coupled high affinity IgE receptor, FcεRI. C5L2-transfected RBL cells were incubated with IgE/DNP and activated by addition of 100 ng/ml HSA-DNP. Pretreatment for 10 min with 100 nM C5a, C3a, C4a, and C5adR caused small but significant increases in the secretory response to HSA-DNP (Fig. 4). Untransfected cells did not

show any increased response to HSA-DNP (Fig. 4), and the pretreatment of C5L2-transfected cells with pertussis toxin at a dose that could completely inhibit the degranulatory response to ligation of CD88 (10 ng/ml for 4 h (30)) also inhibited the effects of C5L2 ligands on the HSA-DNP response (Fig. 4). It appears that a low level of pertussis toxin-sensitive G protein-dependent signal transduction can occur following ligand binding to C5L2. The relatively weak coupling of C5L2 to G protein (probably Gαi) is not surprising because C5L2 does not have a sequence corresponding to the DRY motif found in most chemotactrant and chemokine receptors (Fig. 1); CD88 has 132DRF, C3aR has DRC, but C5L2 has DLC (Fig. 1). The arginine residue of this motif in particular has been shown to be important in coupling to G proteins; mutation of the analogous residue in formyl peptide receptor inhibits signaling because of uncoupling from G protein (27). In addition, the third intracellular loop of C5L2 is much shorter than that of CD88 and C3aR (Fig. 1) and lacks Ser/Thr residues that may be protein kinase C phosphorylation sites as well as a conserved basic region (239KTLK in CD88). Mutation of these Ser/Thr residues to Ala in CD88 inhibits signaling but not ligand binding (18), suggesting that this loop plays an essential role in G protein coupling. RBL cells are regarded as an excellent model system for the expression of granulocyte chemotactrant receptors (28) with similar G proteins and other receptor-associ-
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TABLE I

| Unlabeled ligand | CD88 (I25I-C5a) | C3aR (I25I-C3a) | C5L2 (I25I-C5a) | C5L2 (I25I-C3a) |
|------------------|----------------|----------------|----------------|----------------|
|                  | -log IC50 ± S.E. | IC50 n | -log IC50 ± S.E. | IC50 n | -log IC50 ± S.E. | IC50 n | -log IC50 ± S.E. | IC50 n |
| C5a              | 7.72 ± 0.03     | 19.0 14      | 5.54 ± 0.07     | 2,900 3     | 8.02 ± 0.04**  | 9.50 7      | 6.53 ± 0.16*** | 293 3     |
| C5adR74          | 6.39 ± 0.09     | 412 6       | 5.02 ± 0.18     | 9,670 3     | 7.44 ± 0.10*** | 36.5 3     | <4            | 3         |
| C4a              | 5.35 ± 0.09     | 4,440 4     | 6.60 ± 0.08     | 250 3       | 5.16 ± 0.10**  | 6,860 4     | 6.31 ± 0.12**  | 485 4     |
| C3a              | 4.64 ± 0.33     | 25,100 3    | 6.81 ± 0.05     | 155 12      | 4.64 ± 0.17**  | 22,200 3    | 6.72 ± 0.12**  | 190 7     |

* IC50, concentration of unlabeled ligand resulting in 50% of maximal radioligand binding.

** n, number of separate experiments performed in triplicate.

Fig. 4. Potency of C5L2 ligands of the degranulation response to cross-linkage of the high affinity IgE receptor. RBL cells transfected with C5L2 or untransfected control cells were incubated overnight with 1 μg/ml IgE2DNP and then treated with buffer or 100 nm C5a, C5adR74, C4a, or C3a for 15 min prior to the addition of the cross-linking agent HSA-DNP at 100 ng/ml. Degranulation was assessed as the secretion of β-hexosaminidase. In some cases cells were pretreated with 10 ng/ml pertussis toxin (PT) for 4 h prior to the addition of C5L2 ligands. Results are shown as a percentage of the release stimulated by 100 ng/ml H11006. S.E. IC50

Fig. 5. Ligand-dependent internalization of chemoattractant receptors. Transfected RBL cells were incubated with the stated ligands at 37 °C for 10 min. After quenching in ice-cold buffer, surface expression of receptors was measured by adding antibodies specific for the N termini of CD88 and C3aR and the N-terminal HA tag of C5L2 and quantifying bound antibody levels by flow cytometry. The results are shown as a percentage of the untreated control cell expression and are the means ± S.E. of three separate experiments performed in duplicate. Significantly different from control (= 100): *, p < 5%; **, p < 0.5% (one sample t test). NA, no addition.

involved in anaphylatoxin clearance but might act as a reservoir of cell surface-associated anaphylatoxin to aid chemotaxis or to buffer anaphylatoxin concentrations during an inflammatory response. Alternatively, although internalization and receptor desensitization are not directly correlated, the retention of ligated C5L2 at the cell surface may be involved in prolonging signaling beyond the rapid responses normally stimulated by C5a.

In conclusion, we have shown that C5L2 has high affinity binding sites for C5a and C5adR74 and also binds C3a and C4a with a similar affinity to C3aR. However, C5L2 couples poorly to the intracellular signaling and internalization machinery used by other chemoattractant receptors. The functions of this novel anaphylatoxin-binding protein remain to be defined.

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The Orphan Receptor C5L2 Has High Affinity Binding Sites for Complement Fragments C5a and C5a des-Arg

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