Biochem. J. (1992) 288, 911-917 (Printed in Great Britain)

Cloning and functional expression of the canine anaphylatoxin C5a receptor

Evidence for high interspecies variability

Jason J. PERRET,* Eric RASPE,* Gilbert VASSART*† and Marc PARMENTIER*
*I.R.I.B.H.N., Faculté de Médecine, Université Libre de Bruxelles, Campus Erasme, Bat. C, 808 Route de Lennik, 1070 Bruxelles, Belgium, and †Service de Génétique Médicale, Hôpital Erasme, 808 route de Lennik, 1070 Bruxelles, Belgium

A cDNA clone, DTJP03, encoding an orphan receptor, was isolated from a canine thyroid library, and found to exhibit 68.6% amino-acid identity with the recently described human C5a receptor. This relatively low similarity first suggested that DTJP03 encoded either a C5a receptor subtype, or the presumably related C3a receptor. Binding studies performed on membranes from COS-7 cells expressing the recombinant receptor demonstrated that DTJP03 encoded a high-affinity C5a receptor, with a K_d of 1.2 nM. C3a was unable to compete for C5a binding. Intracellular free calcium concentrations were measured by Quin-2 fluorescence assays in Chinese hamster ovary cells stably transfected with the canine C5a receptor. C5a addition elicited an increase in the intracellular calcium concentration. Extracellular EGTA partially prevented this response, suggesting that activation of the C5a receptor promotes both the release of calcium from intracellular stores, and the influx of extracellular calcium. Genes encoding C5a-receptor subtypes were subsequently searched for by PCR in genomic DNA from human, canine, rat and bovine sources. The result was the amplification of a single gene fragment from each species, with about 70% identity between any two of them. The canine C5a receptor has therefore to be considered as orthologous to the human C5a receptor described previously. The low similarity between C5a receptors from different mammalian species is quite unusual for a G-protein-coupled receptor.

INTRODUCTION

In the course of complement activation, specific cleavage of the C5 component releases the anaphylatoxin C5a, a 74 amino-acid peptide (Fernandez & Hugli, 1978; Hugli, 1981). Binding of C5a to its specific membrane receptor induces physiological responses in a variety of cell types. *In vivo*, C5a is a potent mediator of the acute inflammatory response. *In vitro*, C5a exerts chemotaxis on macrophages and polymorphonuclear leucocytes and is a powerful stimulator of neutrophil function. It induces exocytosis of lysosomal hydrolytic enzymes, enhances production of superoxide radicals, promotes neutrophil leukotriene B_4 synthesis, as well as aggregation, adherence and neutrophil margination. In addition, C5a has spasmogenic effects, stimulating smooth muscle contraction, increasing vascular permeability and promoting mast-cell degranulation and histamine release. C5a also induces serotonin release from platelets, enhances interleukin-1 (IL-1) secretion from macrophages and up-regulates surface expression of the complement receptors CR1 and CR3 (for review see Fearon & Wong, 1983; Goldstein, 1988; Franck & Fries, 1991).

It was known that the C5a receptor belonged to the G-protein-coupled family, but different G-proteins and intracellular pathways seem to be involved in the signal transduction. Most of the effects induced by C5a are mediated through coupling of the C5a receptor to pertussis toxin (PT)-sensitive G-protein(s) (Warner *et al.*, 1987; Nourshargh & Williams, 1990; Rollins *et al.*, 1991). Some effects are, however, insensitive to PT treatment (Monk & Banks, 1991*a*,*b*). The requirement for extracellular calcium also depends on the assay system (Zimmerli *et al.*, 1990; Dore *et al.*, 1990; Kernen *et al.*, 1991).

Using degenerate primers corresponding to the conserved transmembrane segments of the G-protein-coupled-receptor superfamily, we have isolated by PCR (Saiki *et al.*, 1988) a series of orphan receptors from either cDNA or genomic DNA (Libert *et al.*, 1989; Parmentier *et al.*, 1989). Some of these orphan receptors have since been identified (Maenhaut *et al.*, 1990; Libert *et al.*, 1991; Parmentier *et al.*, 1992).

Recently, the cloning of the human C5a receptor was reported (Gerard & Gerard, 1991; Boulay *et al.*, 1991) and sequence comparison revealed a 68.6% identity with one of our orphan receptors, DTJP03. In this paper we present results confirming that DTJP03 encodes the canine C5a receptor. In the search of potential subtypes, partial clones encoding the canine, human, rat and bovine C5a receptors were amplified by PCR. A single type was obtained for each species, sharing about 70% identity with one another. This represents a surprisingly high interspecies variability as compared with other G-protein-coupled receptors.

MATERIALS AND METHODS

Cloning and sequencing

PCR was performed on 0.15 μg of purified cDNA as described previously (Libert *et al.*, 1989; Parmentier *et al.*, 1989), and the amplification product was cloned in M13 vectors for sequencing. JP03, a 600 bp fragment encoding part of an orphan receptor, was used to screen a canine thyroid λgt11 cDNA library (Lefort *et al.*, 1989). The two positive clones were purified to homogeneity and the *Eco*RI cDNA inserts were subcloned in pBluescript SK+ plasmid vector (Stratagene). After subcloning of overlapping restriction fragments in M13mp18 and 19, the clones were sequenced on both strands by the dideoxynucleotide-chain-termination method (Sanger *et al.*, 1977), using an automated DNA sequencer (Applied Biosystems 370A).

The coding region was cloned as a 1350 bp *Eco*RI–*Pst*I fragment in pBluescript SK+ and further subcloned as a

Abbreviations used: IL-1 interleukin 1; IL-8, interleukin 8; PT, pertussis toxin; CHO, Chinese hamster ovary; fMLP, formyl Met-Leu-Phe; ICL, intracellular loops.

```
                                                                     -15  CCGGGAGACCTGAAC   -1

   1  ATGGCCTCCATGAATTTCAGCCCCCCCGAGTACCCCGACTATGGCACTGCCACCCTGGACCCCAACATATTTGTGGATGAGTCTCTCAAC   90
   1   M  A  S  M  N  F  S  P  P  E  Y  P  D  Y  G  T  A  T  L  D  P  N  I  F  V  D  E  S  L  N    30
                                *                             *
                              ------------------------------ I ------------------------------
  91  ACCCCCAAGCTGTCGGTCCCGGATATGATCGCCCTGGTAATCTTCGTGATGGTCTTTCTGGTGGGGGTGCCAGGCAACTTCCTGGTGGTC  180
  31   T  P  K  L  S  V  P  D  M  I  A  L  V  I  F  V  M  V  F  L  V  G  V  P  G  N  F  L  V  V    60
                                *                             *
      -----                      --------------------------- II ---------------------------
 181  TGGGTGACGGGTTTCGAGGTCCGGCGAACCATCAATGCCATCTGGTTTCTCAACCTGGCGGTGGCCGATCTCCTGTCCTGCCTGGCGCTG  270
  61   W  V  T  G  F  E  V  R  R  T  I  N  A  I  W  F  L  N  L  A  V  A  D  L  L  S  C  L  A  L    90
                                *                             *
      ---------                                                   ---------------------------
 271  CCCATCCTGTTTTCGTCCATCGTCCAGCAGGGCTACTGGCCCTTTGGCAACGCTGCCTGCCGCATCCTGCCCTCGCTCATCCTGCTCAAC  360
  91   P  I  L  F  S  S  I  V  Q  Q  G  Y  W  P  F  G  N  A  A  C  R  I  L  P  S  L  I  L  L  N   120
                                *                             *
      ----- III ----------------------
 361  ATGTACGCCAGCATCTTGCTCCTGACCACCATCAGCGCCGACCGCTTTGTCTTGGTGTTTAATCCCATCTGGTGCCAGAACTACCGAGGG  450
 121   M  Y  A  S  I  L  L  L  T  T  I  S  A  D  R  F  V  L  V  F  N  P  I  W  C  Q  N  Y  R  G   150
                                *                             *
           ------------------------------ IV ------------------------------
 451  CCCCAGCTGGCCTGGGCGGCCTGCAGCGTGGCCTGGGCCGTGGCCCTGCTGCTGACCGTACCCTCGTTCATCTTCCGCGGGGTGCACACG  540
 151   P  Q  L  A  W  A  A  C  S  V  A  W  A  V  A  L  L  L  T  V  P  S  F  I  F  R· G  V  H  T   180
                                *                             *
                                                                               ------------
 541  GAGTACTTTCCGTTCTGGATGACGTGCGGCGTGGACTACAGCGGGGTCGGGGTCCTGGTGGAGAGGGGCGTGGCCATCCTCCGGCTGCTC  630
 181   E  Y  F  P  F  W  M  T  C  G  V  D  Y  S  G  V  G  V  L  V  E  R  G  V  A  I  L  R  L  L   210
                                *                             *
      ---------------- V ----------------------------
 631  ATGGGCTTCCTGGGGCCGCTGGTGATACTGAGCATCTGCTACACCTTCCTCCTGATTCGGACCTGGAGCCGGAAGGCCACCCGCTCCACC  720
 211   M  G  F  L  G  P  L  V  I  L  S  I  C  Y  T  F  L  L  I  R  T  W  S  R  K  A  T  R  S  T   240
                                *                             *
               ------------------------- VI ----------------------------
 721  AAGACGCTCAAGGTGGTGGTGGCCGTGGTGGTCAGCTTCTTTGTCCTCTGGCTGCCCTACCAGGTGACGGGGATGATGATGGCTTTGTTC  810
 241   K  T  L  K  V  V  V  A  V  V  V  S  F  F  V  L  W  L  P  Y  Q  V  T  G  M  M  M  A  L  F   270
                                *                             *
                                                ----------------- VII ----------------
 811  TACAAGCACTCGGAAAGCTTCAGACGCGTGTCCCGCCTGGACTCGCTGTGCGTGGCCGTGGCTTACATCAACTGCTGCATAAACCCCATC  900
 271   Y  K  H  S  E  S  F  R  R  V  S  R  L  D  S  L  C  V  A  V  A  Y  I  N  C  C  I  N  P  I   300
                                *                             *
      ---------------
 901  ATCTACGTGCTGGCCGCCCAGGGGTTCCACAGCCGCTTCCTCAAGTCGCTGCCCGCCAGGCTCCGGCAGGTGCTGGCCGAGGAGTCCGTG  990
 301   I  Y  V  L  A  A  Q  G  F  H  S  R  F  L  K  S  L  P  A  R  L  R  Q  V  L  A  E  E  S  V   330
                                *                             *

 991  GGCCGGGACAGCAAGTCTATCACGCTCTCCACGGTGGACACCCCAGCGCAGAAGAGCCAGGGGGTGTGAGGGGGGCCTCCCGGCTTCCTC 1080
 331   G  R  D  S  K  S  I  T  L  S  T  V  D  T  P  A  Q  K  S  Q  G  V                           352
                                *                             *

1081  CTCCCACCTGTCCCTTCCCCCTCTGCTGATTTTAACCCAGCCCCTGGTGGGGAGCTGGCCTGCGAGCGAACTCCCCTCCATTCCTTCCTT 1170

1171  CTTCCCCGCACCCGTCCTCCCTCACTTCCAGGATGAACGCGTCCCTCTGGGACCCTCAACTTTCCTTTTCCATCTGAGGACTTTGGGAAA 1260

1261  AAAGCAGAAACCAGGATTACTCGGAGCACCTCCGAATAGGGCTTATGTCTGCTGCAGGTGGAGATTGTGCATGGGACACAGTCGATACAT 1350

1351  AGAAAAGAGACAAATAGGAACATTCTAACCTTGGGGTGCCTGGGTGGCTCGGTTAAGCATCTGCCTTAGGCTCAGGTCATGATCCCAGGG 1440

1441  TCCTGGGTTCGAGTCCTGCGTCGGGCTCCCCGCAGAGCCTGCTTCTCTGTCTGCCTGTGTCTCTGCATCTCTCTCTGTGTCTCTCATGAA 1530

1531  TAAATAAATAGAAATATTTAAAAAAATGAATGAATACAATCTTAAAGAAAAAGAAAGAAACAGTCTAACCTTTAAAAAAATGGTGATGTG 1620

1621  TTTATTTTACAGAGACCTGGGGGAAAAAAAACCTAAGGAGCATCTCAAAAAGTATTCTTGTGACAGACAAAGCAAAATTCACTGGCTAAG 1710

1711  CTTTTCTTTTCTTTTTCTTTTTTCTTTTTTTTTTTTTTGGCTAAGCTTTTCTGAGTTGATTAAAAAAAAAAAAAACAGTAAGTGAAGGTC 1800

1801  TTACTAATCGGGTTTTGAGAAAACATATTCAGTAAAAAGATGTTCAATGGATTTGGCGAAAAGCAAGTGTGAGGACGGAGTGCCGAAGGG 1890

1891  AGAGGCTGGGAAACATTGGTTAGGCACCTCGGGGGAAGGCGCTCATGGGGAAATTTGACTTTTGACTTTGTAAACTGCTGTTTTGTGG   1978
```

Fig. 1. Nucleotide and deduced amino-acid sequences of the canine C5a receptor cDNA

Numbering is relative to the putative initiation codon which presents a satisfactory sequence context according to Kozak (1989). The putative transmembrane segments are indicated by roman numerals I to VII. * indicates every tenth amino acid.

*Bam*hI–*Xho*I fragment in the eukaryotic expression vector pSVL (Pharmacia).

Sequence handling and data analysis were carried out using DNASIS/PROSIS software (Hitachi), LWL85 software (Li & Luo, 1985) and the GCG/VMS software package (Genetic Computer Group, WI, U.S.A.).

Transfections and cell cultures

The pSVL/DTJP03 construct was transfected in COS-7 cells as described previously (Gérard *et al.*, 1991). Chinese hamster ovary (CHO) CHO-K1 cells were co-transfected with pSVL/DTJP03 and pSV_2Neo as described (Perret *et al.*, 1990). After 10 days of G418 selection, resistant cells were pooled and stored in liquid nitrogen until needed. COS-7 and CHO-K1 cells were cultured using Dulbecco's modified Eagle's medium and Ham's F12 medium respectively, as described previously (Perret *et al.*, 1990). Clonal CHO cell lines were produced by low-density-culture seeding and harvesting of individual clones using sterile rings.

Membrane preparations

COS-7 cell membranes were prepared 72 h after transfection. CHO-K1 cells were prepared using nearly confluent cultures. Membrane preparation was as previously described (Perret *et al.*, 1990). Protein content was determined using the Bradford assay (Bradford, 1976).

Ligands

Human C5a, human C3a, ^{125}I-labelled human C5a and ^{125}I-labelled human C3a were kindly provided by Dr. Bitter-

Table 1. Nucleotide and amino-acid identity scores and synonymous (k_s) and non-synonymous (k_a) evolutionary rates for the C5a receptor and several other members of the G-protein-coupled-receptor family

K_s and k_a values calculated using LWL85 software (Li & Luo, 1985). Bovine and Rat C5a receptor sequences are partial sequences extending from transmembrane segments II to V and II to VII respectively.

Receptor	Species		Evolutionary rates		Identity (%)	
			k_s/year	k_a/year	Nucleotide	Amino acid
Complement C5A	HUM	DOG	2.7	1.3	75.2	68.6
	HUM	RAT	6.1	1.3	73.6	68.1
	HUM	BOV	3.0	0.9	80.5	76.4
	DOG	RAT	6.1	1.6	72.4	64.3
	DOG	BOV	2.6	1.3	78.9	68.2
	RAT	BOV	5.4	1.3	73.2	67.6
Tachykinin NK2	HUM	RAT	3.7	0.5	86.5	88.4
Dopamine D2	HUM	MUS	2.8	0.1	90.7	96.0
B2 Adrenergic	HUM	MUS	3.8	0.4	83.0	85.9
	HUM	RAT	3.3	0.4	81.0	86.4
TSH	HUM	RAT	3.9	0.5	84.4	85.7
	DOG	RAT	4.6	0.4	85.1	89.0
	DOG	HUM	2.5	0.4	89.8	89.7
Muscarinic M1	HUM	PIG	2.4	0.0	92.4	99.1
	HUM	RAT	2.6	0.0	91.4	98.7
	HUM	MUS	2.9	0.1	90.2	98.0
	MUS	PIG	4.0	0.1	88.0	97.2
	PIG	RAT	3.4	0.1	89.4	97.8

Suermann and coworkers (Institut für Medizinische Mikrobiologie, Medizinische Hochschule, Hannover, Germany).

Binding assays

All assays were carried out in 5-ml polypropylene tubes in a final volume of 100 μl, and incubated for 1 h with constant shaking at room temperature. The assay was initiated by the addition of membranes (75–100 μg of protein) to the tubes containing 20 mM-Hepes (pH 7.4)/125 mM-NaCl/5 mM-KCl/0.5 mM-glucose/0.25% (w/v) BSA/1 mM-$CaCl_2$/1 mM-$MgCl_2$ and the ligands. Concentration of labelled ligands in displacement experiments were 1–5 nM ^{125}I-labelled human C5a and 10 nM ^{125}I-labelled human C3a. Non-specific binding was determined by adding an excess of either human C5a (1 μM) or human C3a (10 μM). The assay was terminated by centrifuging (13000 **g**, 0 °C, 10 min) the membranes through a 10% (w/v) sucrose cushion in phosphate-buffered saline. The tubes were then frozen in liquid nitrogen for 2–3 min, and the bottom of each tube containing the membrane pellet was cut with a blade and counted in a gamma counter.

Calcium assay

CHO cell cultures, preparation for assay, and assay conditions were carried out as described previously (Van Sande *et al.*, 1990). Human C5a was used at a concentration of 100–150 nM. To assay the contribution of extracellular calcium, EGTA (1.5 mM) was added to the assay buffer in some experiments.

Genomic PCR

Aliquots (1 μg) of canine, human, rat and bovine genomic DNA were used as a target DNA in PCR reactions (30 cycles of 1 min at 93 °C, 2 min at 55 °C, 3 min at 72 °C). Other annealing temperatures were tested: 52 °C, 50 °C, 48 °C and 45 °C. All other conditions were as for PCR on the canine thyroid cDNA library described above. Primers containing *Xba*I or *Hin*dIII restriction sites for cloning were as follows:

P1 = TAGATCTAGATCAA(T/C)GC(G/C)AT(C/A/T)-TGGTT(T/C)CT;

P2 = ACTTAAGCTT(T/G)ATGCAGCA(G/A)TT(C/A/T)-AT(G/A)TA;

P3 = TAGATCTAGACTGTTTTCGTCCATCGTCCA;

P4 = ACTTAAGCTTACCAC(G/C)ACCTT(C/T)-AGTGT(C/T)TT.

RESULTS AND DISCUSSION

Numerous discrete bands were obtained in PCR reactions, using cDNA from a canine thyroid λgtll library (Lefort *et al.*, 1989) as target DNA, and degenerate primers corresponding to the conserved regions of the second, third, and seventh transmembrane segments of known G-protein-coupled receptors, as described previously (Libert *et al.*, 1989; Parmentier *et al.*, 1989). These bands were cloned into the M13 vectors and sequenced. Open reading frames presenting similarities with G-protein-coupled receptors were searched for in all frames. Three clones encoding new putative members of the receptor superfamily were used as probes to screen the canine thyroid cDNA library. DTJP03, a 600 bp PCR clone, produced two positive signals out of 10^6 clones screened. The larger clone (1993 bp) contained entirely the smaller one (1.5 kb). Sequencing revealed a 1056 bp open reading frame (Fig. 1), having two potential AUG initiation codons (bases 1 and 10). The region surrounding the first AUG is closer to the consensus for initiation sites, as described by Kozak (1989). The coding sequence of DTJP03 was cloned as a 1350 bp insert into pBluescript SK+ and in the pSVL eukaryotic expression vector.

The cDNA encodes a 352 amino-acid protein (Fig. 1) with a

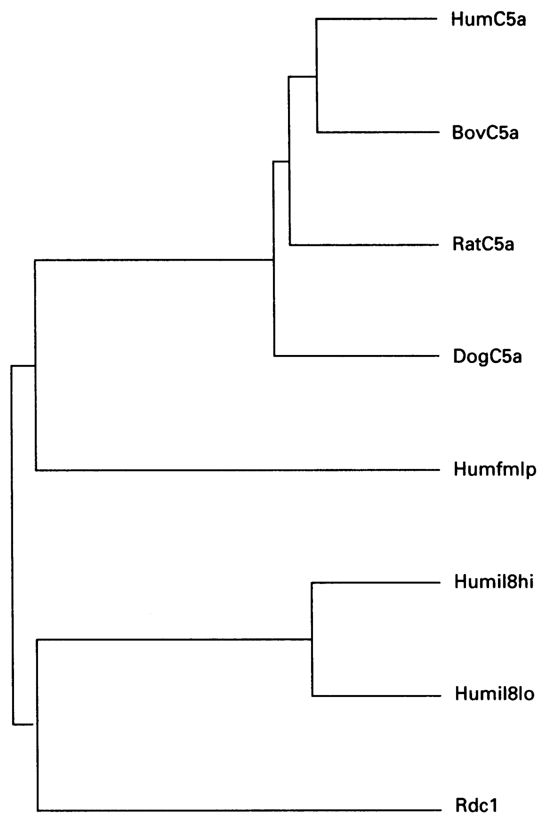

Fig. 2. Dendrogram showing the relative sequence similarities between the C5a receptor from four mammalian species, and the related receptors from the G-protein-coupled family

The dendrogram was generated by using the GCG Pileup software. Abbreviations: HumC5a, human C5a; BovC5a, bovine C5a; RatC5a, rat C5a; DogC5a, dog C5a; Humfmlp, human fMLP receptor; Humil8hi, human IL-8 high-affinity receptor; Humil8lo, human IL-8 low-affinity receptor; Rdc1, our orphan receptor (Libert *et al.*, 1989).

calculated relative molecular mass of 39186. The hydropathy profile (Kyte & Doolittle, 1982) of the deduced amino-acid sequence is consistent with the presence of seven transmembrane domains (results not shown). Sequence comparison with the recently cloned human C5a receptor (Gerard & Gerard, 1991; Boulay *et al.*, 1991) revealed a 68% amino-acid identity. This percentage is well below the identity scores obtained between mammalian orthologues for other G-protein-coupled receptors, which generally ranged between 85 and 98%. A few examples are given for comparison in Table 1. From the dendrogram displayed in Fig. 2, the similarity is of the same order as that observed for the two recently cloned high- and low-affinity interleukin-8 (IL-8) receptors (Holmes *et al.*, 1991; Murphy & Tiffany, 1991). Given this moderate similarity, our first hypothesis was that DTJP03 encoded a receptor closely related to, but different from the published human C5a receptor. We considered that it could encode either a C5a subtype, or the presumably related C3a receptor as suggested by the similar structure of the two ligands (Greer, 1986).

In order to assay the binding characteristics of DTJP03, membranes were prepared from transfected COS-7 cells transiently expressing the recombinant receptor. Transfected COS membranes exhibited a high binding capacity for ^{125}I-labelled human C5a (8000 c.p.m.). Over 80% of the total binding was specifically displaced by a 10^3-fold excess of unlabelled C5a, a 10^4-fold excess of unlabelled C3a being without effect (Fig. 3*a*).

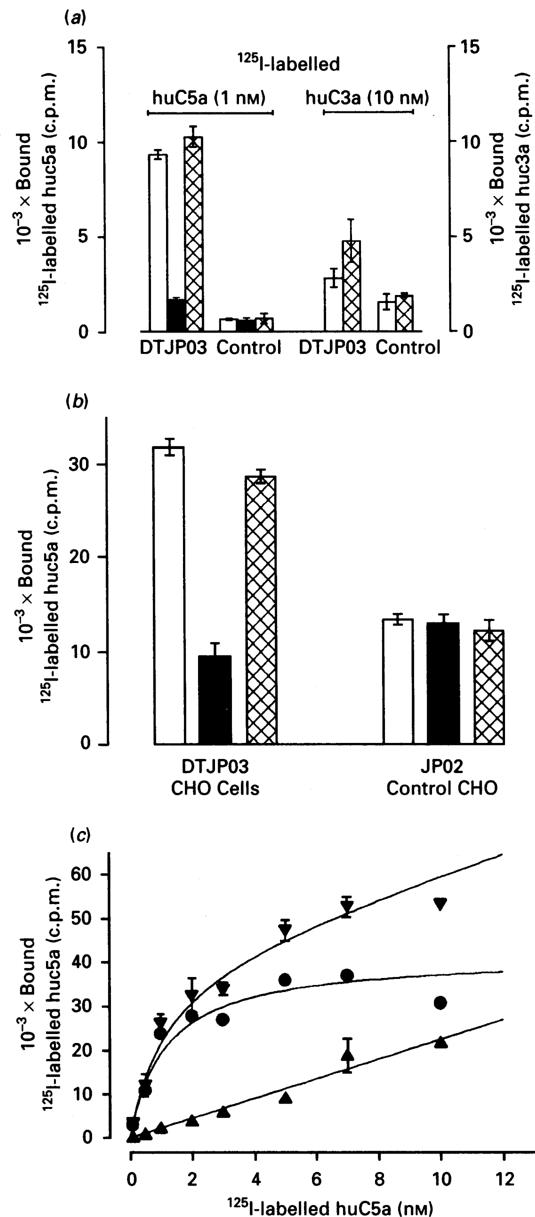

Fig. 3. Binding studies on the canine C5a receptor expressed in COS-7 and CHO cell lines

(*a*) Binding of ^{125}I-labelled human C5a (huC5a) and ^{125}I-labelled human C3a (huC3a) to COS-7 cells transiently expressing DTJP03, and to control COS-7 cells. Bound radiolabelled ligand (open bars) was displaced by an excess of either unlabelled huC5a (1 μM) (■) or huC3a (10 μM) (⊠). (*b*) A similar experiment was performed on CHO cells stably transfected with DTJP03, as compared with CHO cells transfected with the pSV_2Neo plasmid alone (JP02 control). (*c*) Saturation binding experiment performed on COS-7 cells transiently expressing the canine C5a receptor. Total (▼), specific (●) and non-specific binding (▲) are represented. The non-specific binding was determined in the presence of 1 μM unlabelled huC5a. Curve fitting using a non-linear regression algorithm yielded an apparent K_d of 1.2 nM.

No specific binding of ^{125}I-labelled human C3a was obtained with transfected cells. Control COS cell membranes did not display significant binding for either C5a (700 c.p.m.) or C3a. Saturation curves obtained with transfected COS cell membranes and increasing concentrations of ^{125}I-labelled human C5a demonstrated a saturable high-affinity binding site with an apparent K_d of 1.2 nM (Fig. 3*c*), this being within the range

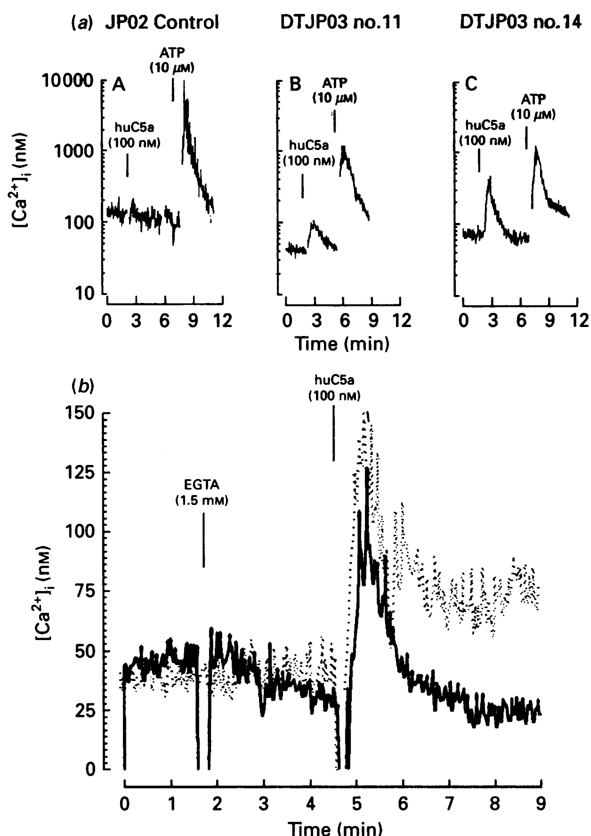

Fig. 4. Intracellular calcium measurements in stably transfected CHO cells and JP02 control CHO cells

Experiments were performed using a Quin-2 fluorescence assay and a SPEX fluorimeter. (*a*) Panels A, B and C represent respectively the responses elicited by the successive addition of human C5a (huC5a) (100 nM) and ATP (10 μM) on a CHO cell line transfected with pSV$_2$Neo (JP02 control), and two clonal cell lines (nos. 11 and 14) transfected with DTJP03, and expressing the C5a receptor with a different efficiency. (*b*) Effect of the extracellular calcium concentration on the response to C5a in clone no. 14. The dotted line represents the response to huC5a (100 nM) in control medium containing 1 mM-Ca^{2+}. The continuous line represents the response to huC5a after addition of an extracellular excess of EGTA (1.5 mM).

previously described for the human neutrophil and macrophage C5a receptors (Chenoweth *et al.*, 1982; Huey & Hugli, 1985; Yancey *et al.*, 1989).

CHO-K1 cells were co-transfected by the pSVL/DTJP03 construct and the pSV$_2$Neo vector conferring resistance to neomycin. After selection by G418, the pool of resistant clones was used to prepare membranes. Specific binding of ^{125}I-labelled human C5a to stably transfected CHO cell-membrane preparations yielded results similar to those obtained with COS-7 cells (Fig. 3*b*), while C3a was unable to bind to the receptor. The pool of neomycin-resistant CHO cells was cloned by high-dilution-culture seeding followed by subsequent recovery of the isolated colonies. The individual clones were screened by measuring intracellular calcium concentrations by the Quin-2 fluorescence assay (Van Sande *et al.*, 1990). Only 25 % (6/24) of the clones responded to 150 nM-human C5a in the Ca^{2+} assay. One clone (No. 14) presented a large response to C5a, as compared with that elicited by 10 μM-ATP, used as positive control (Fig. 4*a*). This clone was used for further studies. Addition of 1.5 mM-EGTA to the extracellular assay medium modified the calcium fluorescence signal induced by subsequent addition of the agonist (Fig. 4*b*). In the presence of extracellular calcium, the intracellular calcium concentration increased rapidly to its maximal level within 1 min, then decreased gradually back to the basal level or slightly higher. However, when extracellular calcium was chelated by excess EGTA, human C5a evoked a sharp transient increase of intracellular calcium, that rapidly fell off to beneath the basal level. As the initial increase in calcium concentration is resistant to extracellular calcium depletion by EGTA, it is likely that calcium is released from intracellular stores. On the other hand, the subsequent decrease in calcium concentration is faster in the absence of extracellular calcium, suggesting that calcium influx contributes to the sustained phase. This behaviour is typical of receptors coupled to a G-protein activating the inositol phosphate cascade (Kojima, 1990; Dore *et al.*, 1990).

These results clearly demonstrate the DTJP03 is a canine high-affinity C5a receptor that functionally couples to the InsP_3–calcium cascade in stably transfected CHO cell lines. Although there is no pharmacological evidence for C5a receptor subtypes, the low degree of similarity between the canine and the human receptors could be indicative of a possible molecular heterogeneity of the C5a receptor. We therefore searched for orthologues of our canine C5a and of the human C5a receptors in four mammalian species: human, dog, rat and cow. A PCR-based approach was used, in which four nucleotide sequences, conserved between the cloned canine and human C5a receptors, were used as primers to amplify related gene fragments from genomic DNA. Like most of the other members of the seven transmembrane G-protein-coupled-receptor superfamily, the C5a receptor lacks introns in its coding sequence. Four moderately degenerate primers corresponding to parts of transmembrane segments II (P1 and P2), VI (P3) and VII (P4) were defined (see the Materials and methods section). Independent PCR reactions were performed, using all four primer combinations (i.e. P1 versus P2; P1 versus P4; P3 versus P2 and P3 versus P4). To allow primer hybridization in the presence of potential mismatches, several annealing temperatures (55 °C, 52 °C, 50 °C, 48 °C, and 45 °C) were used. Down to 45 °C, only one band was visible for each primer combination at the expected size (results not shown). These bands were cloned into the bacteriophage M13mp18 and 19 vectors and sequences were obtained from five or six clones under each condition. All sequences obtained from canine DNA were identical to DTJP03; likewise all sequences from human genomic DNA were identical to the published human C5a receptor sequence. The bovine and rat sequences were unique as well, regardless of PCR stringency or primer combination. The partial amino-acid sequences obtained from rat and bovine sequences were aligned with the canine and human sequences (Fig. 5). Similarity between any two sequences was close to 70 %. These results demonstrate that our canine receptor and the reported human C5a receptors effectively represent orthologues.

There remains then the question of the surprisingly low interspecies amino-acid conservation (68.9 ± 3.7 %), which is in contrast to the high conservation between other G-protein-coupled receptors, such as the cannabinoid, the thyroid stimulating hormone or the muscarinic receptors. This contrast appears clearly when the evolutionary rates for synonymous changes (k_s) and non-synonymous changes (k_a) (Li & Luo, 1985) are calculated for the C5a receptors and several other members of the G-protein-coupled-receptor family, as well as the nucleotide and amino-acid identities (Table 1). As expected, the k_s values, reflecting the rate of nucleotide substitutions that do not affect amino acids, are relatively constant for all receptors, as it reflects solely the evolutionary distance between species. On the other hand, the k_a values, reflecting the nucleotide substitution rate affecting the amino-acid sequence, are low for most

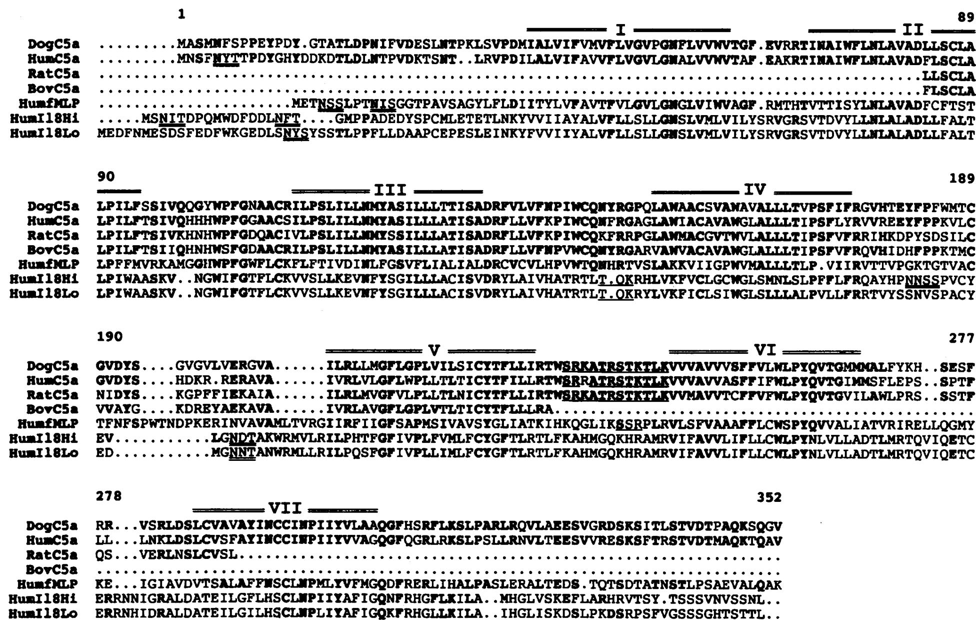

Fig. 5. Amino-acid alignment of the canine and human C5a receptors, the partial rat and bovine sequences obtained from genomic DNA by PCR, and the sequences from the related chemoattractant receptors to human IL-8 and human fMLP

Amino acids identical to the canine C5a receptor are represented in bold characters. Numbering is relative to the canine C5a receptor amino-acid sequence. The transmembrane segments are overlined and numbered I to VII. The putative sites for *N*-linked glycosylation in the *N*-terminal and extracellular domains are underlined twice; the putative sites for phosphorylation by protein kinases A and C in the second and third ICLs are underlined once. The alignment was performed by using the GCG Pileup software. See legend to Fig. 2 for key to abbreviations.

G-protein-coupled receptors, and significantly higher for the C5a receptor. A corollary of these high k_a values is the lower identity at the amino-acid level as compared with the nucleotide level in contrast to the increase observed for the other members of the G-protein-coupled family. This signifies that evolutionary constraints affect quite differently classes of receptors that are believed to share a common transmembrane organization and structure–function relationships. It is unclear to us why the C5a receptor appears to deviate from the strong conservation prevailing in this gene family.

Northern-blot analysis (results not shown) on different canine tissues detected C5a receptor transcripts in thyroid (but not in cultured thyrocytes), testis, brain, lung, kidney, spleen and stomach. This partial distribution, as well as the cloning from a thyroid library, reflects the presence of circulating leucocytes and tissue macrophages in all organs.

Within this background of high interspecies variability, the similarities between protein sequences could pinpoint functionally important conserved segments or residues, implicated either in ligand–receptor interactions, G-protein-coupling or receptor desensitization. We therefore aligned the C5a receptor sequences with their closest relatives of the G-protein-coupled family, the human formyl Met-Leu-Phe (fMLP) (Boulay *et al.*, 1990) and the two IL-8 receptors (Fig. 5). The extracellular *N*-terminal domain is poorly conserved between the canine and human receptors with only 44% (17/39) identical residues. It has been proposed that the high negative charges of the receptor are important for the interaction with the positively charged ligand, although few of these charges are on the surface of the C5a ligand (Zuiderweg *et al.*, 1989; Mollison *et al.*, 1989). Within the *N*-terminal domain of the receptor, only three charged residues are identical in both species, and the same observation prevails for the extracellular loops that are the least conserved parts of the receptor. This poor conservation, together with the absence of species selectivity for the ligand (the canine receptor interacts very efficiently with human C5a), do not give support to the involvement of the extracellular domains in receptor–ligand interactions.

Interestingly, in contrast to the human C5a receptor, the canine receptor does not present potential *N*-glycosylation sites in its extracellular *N*-terminus. The conserved Asn-Xaa-Thr sequence is, in the canine sequence, followed by a proline, which has been shown to prevent glycosylation (Pless & Lennarz, 1977; Bause, 1983). Such differential glycosylation is also observed for the C5a ligand which is glycosylated in human (Zuiderweg *et al.*, 1989) but not in pig (Williamson & Madison, 1990). The intracellular *C*-terminus contains numerous serine and threonine residues (10 for canine; 11 for human) that are potential phosphorylation sites for βARK-related serine/threonine protein kinases (Benovic *et al.*, 1989). Another interesting feature of the *C*-terminus, shared with the bovine NPY (Rimland *et al.*, 1991), human IL-8, RDC1 (Libert *et al.*, 1989), and the human fMLP receptors, is the absence of a cysteine residue, conserved among most G-protein-coupled receptors, thought to serve as a site for palmitoylation (Dohlman *et al.*, 1991).

Potential phosphorylation sites, conserved in the four species, are present in the third intracellular loop: one for the cyclic AMP-dependent kinases (protein kinase A) (Feramisco *et al.*, 1980; Glass *et al.*, 1986) at residue Thr-237, and three for the protein kinase C (Woodgett *et al.*, 1986) at residues Ser-233, Ser-239 and Thr-242. Conserved residues in the C5a receptors include Phe-136 which replaces the tyrosine residue of the Asp-Arg-Tyr tri-peptide motif (end of TM3) common to most G-protein-coupled receptors.

The transmembrane segments are the most conserved, with 73% identity (119/162); however, the intracellular loops (ICL) are also highly conserved with 70% (31/44) of identical residues. The lowest identity is observed for ICL2 (58%, 11/19). ICL3 presents up to 87% (13/15) amino-acid identity within the C5a receptor group. The C5a, fMLP and IL-8 receptors share very little similarity within ICL3, despite the coupling of these receptors to a common intracellular pathway, and the involve-

ment of ICL3 in G-protein coupling (Dohlman *et al.*, 1991; Bonner, 1992). Charged residues thought to interact with negative charges of G-proteins (Dohlman *et al.*, 1991) are, however, present in all cases.

As a conclusion, cloning of the C5a receptors in several species along with the characterization of the corresponding ligands should allow a better approach in determining critical amino acids implicated in receptor–ligand interaction, G-protein-coupling and desensitization. It will pinpoint candidate residues for mutagenesis and chimeric constructions, both in the receptors and the ligands. Ultimately, the availability of the cloned receptors should help the design of pharmacologically active (non-peptide) inhibitors that could be used in syndromes were inappropriate complement activation occurs.

We would like to thank Drs. J. E. Dumont, J. Khöl and A. Klos for useful discussions, Dr. S. Swillens for his help in binding studies and computer analysis of results, Dr. P. Cochaux and C. Mollereau for lending a hand when most needed. We also appreciated the technical help of Y. Mauquoy in maintaining the cell lines. This work was supported by the Belgian programme on Inter-University Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming. It was also supported by the Fonds de la Recherche Scientifique Médicale of Belgium, Boehringer Ingelheim and the Association Recherche Biomédicale et Diagnostic. The scientific responsibility is assumed by the authors. J.P. and E.R. are respectively fellows of the IRSIA and Televie. M.P. is Chercheur Qualifié of the Fonds National de la Recherche Scientifique.

REFERENCES

Bause, E. (1983) Biochem. J. **209**, 331–336

Benovic, J. L., DeBlasi, A., Stone, W. C., Caron, M. G. & Lefkowitz, R. J. (1989) Science **246**, 235–346

Bonner, T. I. (1992) Trends Pharmacol. Sci. **13**, 48–50

Boulay, F., Tardif, M., Brouchon, L. & Vignais, P. (1990) Biochem. Biophys. Res. Commun. **168**, 1103–1109

Boulay, F., Mery, L., Tardif, M., Brouchon, L. & Vignais, P. (1991) Biochemistry **30**, 2983–2990

Bradford, M. (1976) Anal. Biochem. **72**, 248–259

Chenoweth, D. E., Goodman, M. G. & Weigle, W. O. (1982) J. Exp. Med. **156**, 68–78

Dohlman, H. G., Thorner, J., Caron, M. G. & Lefkowitz, R. J. (1991) Annu. Rev. Biochem. **60**, 653–688

Dore, M., Slauson, D. O., Suyemoto, M. M. & Nielson, N. R. (1990) Inflammation **14**, 71–82

Fearon, D. T. & Wong, W. W. (1983) Annu. Rev. Immunol. **1**, 243–271

Feramisco, J. R., Glass, D. B. & Krebs, E. G. (1980) J. Biol. Chem. **255**, 4240–4245

Fernandez, H. N. & Hugli, T. E. (1978) J. Biol. Chem. **253**, 6955–6964

Franck, M. M. & Fries, L. F. (1991) Immunology Today **12**, 322–326

Gerard, N. P. & Gerard, C. (1991) Nature (London) **349**, 614–617

Gérard, C. M., Mollereau, C., Vassart, G. & Parmentier, M. (1991) Biochem. J. **279**, 129–134

Glass, D. B., El-Maghrabi, M. R. & Pilkis, S. J. (1986) J. Biol. Chem. **261**, 2987–2993

Goldstein, I. M. (1988) in Inflammation: Basic Principles and Clinical Correlates (Gallin, J. I., Goldstein, I. M. & Synderman, R., eds.), pp. 309–323, Raven Press, New York

Greer, J. (1986) Enzyme **36**, 150–163

Holmes, W. E., Lee, J., Kuang, W. J., Rice, G. C. & Wood, W. I. (1991) Science **253**, 1278–1280

Huey, R. & Hugli, T. E. (1985) Immunology **235**, 2063–2068

Hugli, T. E. (1981) CRC Crit. Rev. Immunol. **1**, 321–366

Kernen, P., Wymann, M. P., von Tscharner, V., Deranleau, D. A., Tai, P. C., Spry, C. J., Dahinden, C. A. & Baggiolini, M. (1991) J. Clin. Invest. **87**, 2012–2017

Kojima, I. (1990) in G Proteins (Iyengor, R. & Birnbaumer, L., eds.), chapt. 21, pp. 503–517, Academic Press, New York

Kozak, M. (1989) J. Cell Biol. **108**, 229–241

Kyte, J. & Doolittle, R. F. (1982) J. Mol. Biol. **157**, 105–132

Lefort, A., Lecocq, R., Libert, F., Lamy, F., Swillens, S., Vassart, G. & Dumont, J. E. (1989) EMBO J. **8**, 111–116

Li, W. H. & Luo, C. C. (1985) Mol. Biol. Evol. **2**, 150–174

Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M.-J., Dumont, J. E. & Vassart, G. (1989) Science **244**, 569–572

Libert, F., Schiffmann, S., Lefort, A., Parmentier, M., Gérard, C. M., Dumont, J. E., Vanderhaeghan, J.-J. & Vassart, G. (1991) EMBO J. **10**, 1677–1682

Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentier, M., Vanderhaeghen, J.-J., Dumont, J. E., Vassart, G. & Schiffmann, S. (1990) Biochem. Biophys. Res. Commun. **173**, 1169–1178

Mollison, K. W., Mandecki, W., Zuiderweg, E. R. P., Fayer, L., Fey, T. A., Krause, R. A., Conway, R. G., Miller, L., Edalji, R. P., Shallcross, M. A., Lane, B., Lawrence-Fox, J., Greer, J. & Carter, G. W. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 292–296

Monk, P. N. & Banks, P. (1991*a*) Biochim. Biophys. Acta **1092**, 251–255

Monk, P. N. & Banks, P. (1991*b*) J. Mol. Endocrinol. **6**, 241–247

Murphy, P. M. & Tiffany, H. L. (1991) Science **253**, 1280–1283

Nourshargh, S. & Williams, T. J. (1990) J. Immunol. **145**, 2633–2638

Parmentier, M., Libert, F., Maenhaut, C., Lefort, A., Gérard, C. M., Perret, J., Van Sande, J., Dumont, J. E. & Vassart, G. (1989) Science **246**, 1620–1622

Parmentier, M., Libert, F., Schurmans, S., Schiffmann, S., Lefort, A., Eggerickx, D., Ledent, C., Mollereau, C., Gérard, C. M., Perret, J., Grootegoed, A. & Vassart, G. (1992) Nature (London) **355**, 453–455

Perret, J., Ludgate, M., Libert, F., Gérard, C., Dumont, J. E., Vassart, G. & Parmentier, M. (1990) Biochem. Biophys. Res. Commun. **17**, 1044–1050

Pless, D. D. & Lennarz, W., J. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 134–138

Rimland, J., Xin, W., Sweetman, P., Saijoh, K., Nestler, E. J. & Duman, R. S. (1991) Mol. Pharmacol. **40**, 869–875

Rollins, T. E., Siciliano, S., Kobayashi, S., Cianciarulo, D. N., Bonilla-Argudo, V., Collier, K. & Springer, M. S. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 971–975

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) Science **239**, 487–491

Sanger, F., Nicklen, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467

Van Sande, J., Raspé, E., Perret, J., Lejeune, C., Maenhaut, C., Vassart, G. & Dumont, J. E. (1990) Mol. Cell. Endocrinol. **74**, R1–R6

Warner, J. A., Yancey, K. B. & MacGlashan, D. W. (1987) J. Immunol. **139**, 161–165

Williamson, M. P. & Madison, V. S. (1990) Biochemistry **29**, 2895–2905

Woodgett, J. R., Gould, K. L. & Hunter, T. (1986) Eur. J. Biochem. **161**, 177–184

Yancey, K. B., Lawley, T. J., Derssokian, M. & Harvath, L. (1989) J. Invest. Dermatol. **92**, 184–189

Zimmerli, W., Reber, A. M. & Dahinden, C. A. (1990) J. Infect. Dis. **161**, 242–249

Zuiderweg, E. R. P., Nettesheim, D. G., Mollison, K. W. & Carter, G. W. (1989) Biochemistry **28**, 172–185

Received 5 June 1992; accepted 22 July 1992