Expression Cloning of a Receptor for C5a Anaphylatoxin on Differentiated HL-60 Cells†

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ABSTRACT: A cDNA clone encoding the human C5a anaphylatoxin receptor has been isolated by expression cloning from a CDM8 expression library prepared from mRNA of human myeloid HL-60 cells differentiated to the granulocyte phenotype with dibutyryladenosine cyclic monophosphate. The cDNA clone was able to transfer to COS-7 cells the capacity to specifically bind iodinated human recombinant C5a. The cDNA was 2.3 kb long, with an open reading frame encoding a 350-residue polypeptide. Cross-linking of iodinated C5a to the plasma membrane of transfected COS cells revealed a complex with an apparent molecular mass of 52–55 kDa, similar to that observed for the constitutively expressed receptor in differentiated HL-60 cells or human neutrophils. Although differentiated HL-60 cells display a single class of binding sites, with a dissociation constant of approximately 800–900 pM, the C5a-R cDNA, expressed in COS cells, generates both high-affinity (1.7 nM) and low-affinity (20–25 nM) receptors. Sequence comparison established that the degree of sequence identity between the C5a receptor and the *N*-formylpeptide receptor is 34%.

The anaphylatoxin peptide C5a is a 74 amino acid glycoprotein that is derived from the complement component C5 during activation of the complement cascade (Hugli, 1981). In vivo, C5a is thought to play a significant role in the inflammatory response and in a number of clinical disorders (Golstein, 1988). In vitro, C5a is a potent activator of neutrophil and macrophage functions, including production of superoxide radicals, chemotaxis, aggregation, adhesiveness, and release of hydrolytic enzymes from secretory granules [for review see Golstein (1988)]. In addition, the anaphylatoxin has been shown to have a spasmogenic effect on various tissues; it stimulates smooth muscle contraction (Stimler et al., 1981; Scheid et al., 1983), induces histamine release from mast cells (Johnson et al., 1975), promotes serotonin release from platelets (Meuer et al., 1981), and increases vascular permeability (Jose et al., 1981).

The responses elicited by C5a in polymorphonuclear leukocytes result from the binding of the anaphylatoxin to a high-affinity receptor on the plasma membrane (Chenoweth & Hugli, 1978, 1980). In these cells, it appears that the mechanism of signal transduction through the membrane involves one or more GTP-binding proteins (G proteins) as is the case with other chemotactic receptors, such as the *N*-formylpeptide receptor (Okajima & Ui, 1984) and the leukotriene B4 receptor (Goldman et al., 1987; Miki et al., 1990). Three sets of observations support this conclusion: (1) the treatment of neutrophils with *Bordetella pertussis* toxin results in the ADP-ribosylation of a 40-kDa membrane protein and in the inhibition of the responsiveness to C5a and other chemoattractants (Becker et al., 1985; Shirato et al., 1988), (2) the binding of C5a to its receptor is inhibited by a variety of guanine nucleotides (Siciliano et al., 1990), and (3) the receptor appears to copurify with a G protein of the Gi class (Rollins et al., personal communication). However, the details of the interactions between the G protein(s) and the C5a receptor are only partially understood.

While it seems likely, on the basis of the similarities between the responses evoked by C5a and by others chemotactic factors such as fMLP,[1] LTB4 and PAF, that the C5a receptor shares common features with these other chemoattractant receptors, very little is known about its structure and biochemical nature. The receptor for C5a has been characterized on human neutrophils and the differentiated human promyelocytic cell line U937. The specific cross-linking of ^{125}I-labeled C5a to the plasma membranes of these cells, either with bifunctional agents or with photoreactive probes, has allowed the identification of a complex with an apparent mass of 52–55 kDa on sodium dodecyl sulfate gels (Rollins et al., 1985; Johnson et al., 1985; Huey & Hugli, 1985; Gerard et al., 1989). However, the difficulties encountered in purifying sufficient quantities of material have prevented further characterization of the C5a receptor.

Using a direct expression cloning strategy similar to that used for cloning the human *N*-formylpeptide receptor (Boulay et al., 1990a), we have isolated a cDNA identified as coding for the human C5a receptor. In this report, we describe the characterization of the recombinant receptor with regard to its binding parameters, cross-linking with ^{125}I-hrC5a, and mRNA analysis. We demonstrate that the human C5a receptor and the human *N*-formylpeptide receptor, of which we have recently elucidated the structure (Boulay et al., 1990a,b), share striking similarities.

Materials and Methods

Cells and Cell Culture. HL-60 and COS-7 cells were from the American Type Culture Collection (Rockville, MD), U937 cells were a gift from Dr. Craig Gerard (Harvard Medical School, Boston, MA). COS-7 monolayers were grown in

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‡ The nucleotide sequence reported in Figure 1 has been submitted to GenBank under accession number J05327.

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[1] Abbreviations: BSA, bovine serum albumin; DSS, disuccinimido suberate; fMLP, *N*-formylmethionine-leucine-phenylalanine; HBS, Hanks' balanced salt solution; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; hrC5a, human recombinant C5a; IL-8, interleukin 8; LTB4, leukotriene B4; PAF, platelet activating factor, PBS, phosphate-buffered saline; Bt2cAMP, N^6,O^2-dibutyryladenosine 3',5' cyclic monophosphate.

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Dulbecco's modified Eagle's medium (DME) containing 10% heat-inactivated fetal calf serum (FCS), 100 μM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. HL-60 and U937 promyelocytic cells were cultured in suspension at a density not exceeding 2×10^6 cells/mL in RPMI 1640 supplemented with 10% heat-inactivated FCS, glutamine, and antibiotics. Differentiation was induced by Bt2cAMP as indicated in the text.

Radioligand. For receptor binding studies and library screening, human recombinant C5a (hrC5a from Sigma) was labeled with ^{125}I by using immobilized lactoperoxidase and glucose oxidase (Enzymobeads from BioRad). One nanomole of hrC5a, diluted with 50 μL of 0.2 M sodium phosphate buffer pH 7.5, and 25 μL of 2% α-D-glucose, was shaken with 2 mCi (1 nmol) of Na ^{125}I (Amersham) and 50 μL of rehydrated enzymobeads. After a 20–25-min period of incubation, the enzymobeads were centrifuged, and the supernatant was quenched with an excess of cold iodine and immediately filtered through a Bio-Gel P6DG column (0.5 × 20 cm) equilibrated in PBS/1% BSA. About 82–85% of the loaded material was recovered in the pass-through fraction. The average specific activity attained varied between 300 and 400 cpm/fmol.

COS-7 Cells Transfection. Subconfluent COS-7 monolayers, seeded the day before in 10-cm tissue culture plates, were transfected with about 2 μg of cDNA/CDM8 plasmid minipreps by a DEAE-dextran protocol as previously described by Seed and Aruffo (1987).

Receptor Binding Assays. All assays were carried out at 4 °C with differentiated HL-60 cells in suspension or with transfected COS-7 cell monolayers. In brief, HL-60 cells grown for 46 h in the presence of 500 μM Bt2cAMP were resuspended in HBS, 1% BSA, and 25 mM HEPES (pH 7.5) at a density of 2×10^6 cells/mL. Aliquots of 10^6 cells were incubated with increasing concentrations of ^{125}I-labeled hrC5a in the presence or absence of 500 nM hrC5a in a final volume of 500 μL for 2 h. Cell suspensions were layered over 500 μL of a chilled solution of sucrose (8% in HBS) and spun for 4 min in an Eppendorf centrifuge. The cell pellets were removed by cutting the tube with a knife blade, and the cell-associated radioactivity was determined by counting in a γ-counter.

Subconfluent monolayers of COS-7 cells in 60-mm tissue culture dishes were transfected by the DEAE-dextran method with 1 μg of CsCl-purified C5a-R cDNA. Forty-eight hours after transfection, monolayers were washed twice with chilled HBS/25 mM HEPES (pH 7.5) and once with HBS/HEPES/1% BSA and incubated in duplicate with increasing concentrations of ^{125}I-hrC5a diluted in 1 mL of ice-cold DME, 1% BSA, and 25 mM HEPES (pH 7.5) in the presence or absence of unlabeled hrC5a (500 nM). Incubations were performed for 2 h with gentle rocking. Monolayers were washed three times at 4 °C and solubilized in 1 M NaOH, and the bound radioactivity was counted in a γ-counter.

Cross-Linking of ^{125}I-hrC5a. The procedure used for cross-linking ^{125}I-hrC5a to recombinant and native C5a receptors on transfected COS cells or Bt2cAMP-treated HL60 cells was similar to that described by Rollins and Springer (1985). The procedure was identical with that of the binding assay through the incubation and washing steps at 4 °C. Bound ^{125}I-hrC5a was cross-linked to the receptor by treating the COS cell monolayer (10^6 cells) or HL-60 cells (2×10^6 cells) with 2 mL of 1 mM disuccinimido suberate (DSS) in ice-cold PBS for 15 min. After addition of 100 μL of 1 M glycine, pH 8, the cells were washed with PBS and incubated in PBS supplemented with 5 mM diisopropyl fluorophosphate for 10 min at 4 °C. COS cell monolayers were scraped from the tissue culture plate with a rubber policeman in 1 mL of PBS containing 1 mM phenylmethanesulfonyl fluoride and 20 μg/mL leupeptin. Pellets of COS or HL-60 cells were resuspended in 60 μL of Laemmli dissociation buffer containing 5 mM dithiothreitol, and disrupted with a sonicator cell disruptor W10 (Ultrasonics, Plainview, NY) for 20 s at setting 6. The insoluble material was centrifuged in an Eppendorf microfuge for 4 min. The supernatants were analyzed under denaturing conditions on a 10% polyacrylamide slab gel with a 5% stacking gel, prepared and run according to Laemmli (1970).

Nucleotide Sequencing. The C5a-R cDNA was sequenced by the dideoxy chain-termination method with primer extension (Sanger et al., 1977) using the modified T7 polymerase (Sequenase, U.S. Biochemicals). Taq polymerase (Pharmacia) and 7-deaza-2′-deoxyguanosine 5′-triphosphate were used at 72 °C to resolve C/G-rich regions and secondary structures (Innis et al., 1988). Both strands were entirely sequenced.

Northern Blot Analysis. Total RNA was isolated from HL-60, U937, and COS cells by the guanidinium isothiocyanate/phenol method at pH 4 as described by Chomczynski and Sacchi (1987). RNAs were fractionated on 1.2% agarose/formaldehyde gels (Maniatis et al., 1982) and transferred to nylon membranes (Hybond N from Amersham) according to the manufacturer's recommendations. Prior to hybridization, the filters were soaked in 5 mL of rapid hybridization buffer (Amersham) at 65 °C. Hybridization was performed in the same buffer for 3 h at 65 °C with the gel-purified restriction fragment *Hin*dIII–*Pst*I (0.35 kb) that encompasses the 5′ untranslated region and first third of the coding region. This probe was labeled with ^{32}P by random priming (Feinberg & Vogelstein, 1983) After hybridization, the nylon membrane was washed twice in 2× SSC/0.1% SDS at room temperature, once in 1× SSC/0.1% SDS for 15 min at 65 °C, and twice in 0.7× SSC/0.1% SDS for 15 min at 65 °C.

Results and Discussion

An unamplified CDM8 cDNA library prepared from mRNA isolated from human myeloid HL-60 cells differentiated to the granulocyte phenotype with Bt2cAMP was screened by using a direct expression cloning strategy in simian COS cells. Three days after the transfection of subconfluent COS cells with DNA from small pools of clones, positive pools were detected by assaying the cells for endocytosis of iodinated hrC5a according to a methodology previously described for cloning the *N*-formylpeptide receptor (Boulay et al., 1990a). While the nonspecific binding of iodinated *N*-formylpeptide to transfected COS cells was low (1800 cpm), the nonspecific binding of 3–4 nM ^{125}I-hrC5a to a 10-cm plate of subconfluent COS cell monolayers yielded a higher background with approximately 9500 cpm ± 800 cpm. The background level was not significantly reduced by an excess of unlabeled hrC5a (Figure 2A), and furthermore, there was a linear relationship between the amount of radioactivity bound to monolayers of nontransfected COS cells and the concentration of ^{125}I-hrC5a added to the plates, which indicated that COS cells lack the C5a receptor. Out of 60 pools of approximately 700 colonies, we identified three pools that reproducibly gave signals 1500–2000 cpm above the background. One of these pools was partitioned into smaller pools until a single clone capable of causing transfected COS cells to bind and to endocytose ^{125}I-hrC5a was ultimately isolated. When 1–2 μg of the pure cDNA was used to transfect COS cell monolayers in a 10-cm tissue culture plate, the cells were able to accumulate 5×10^5 cpm after incubation with 4 nM ^{125}I-hrC5a for 60 min at 37

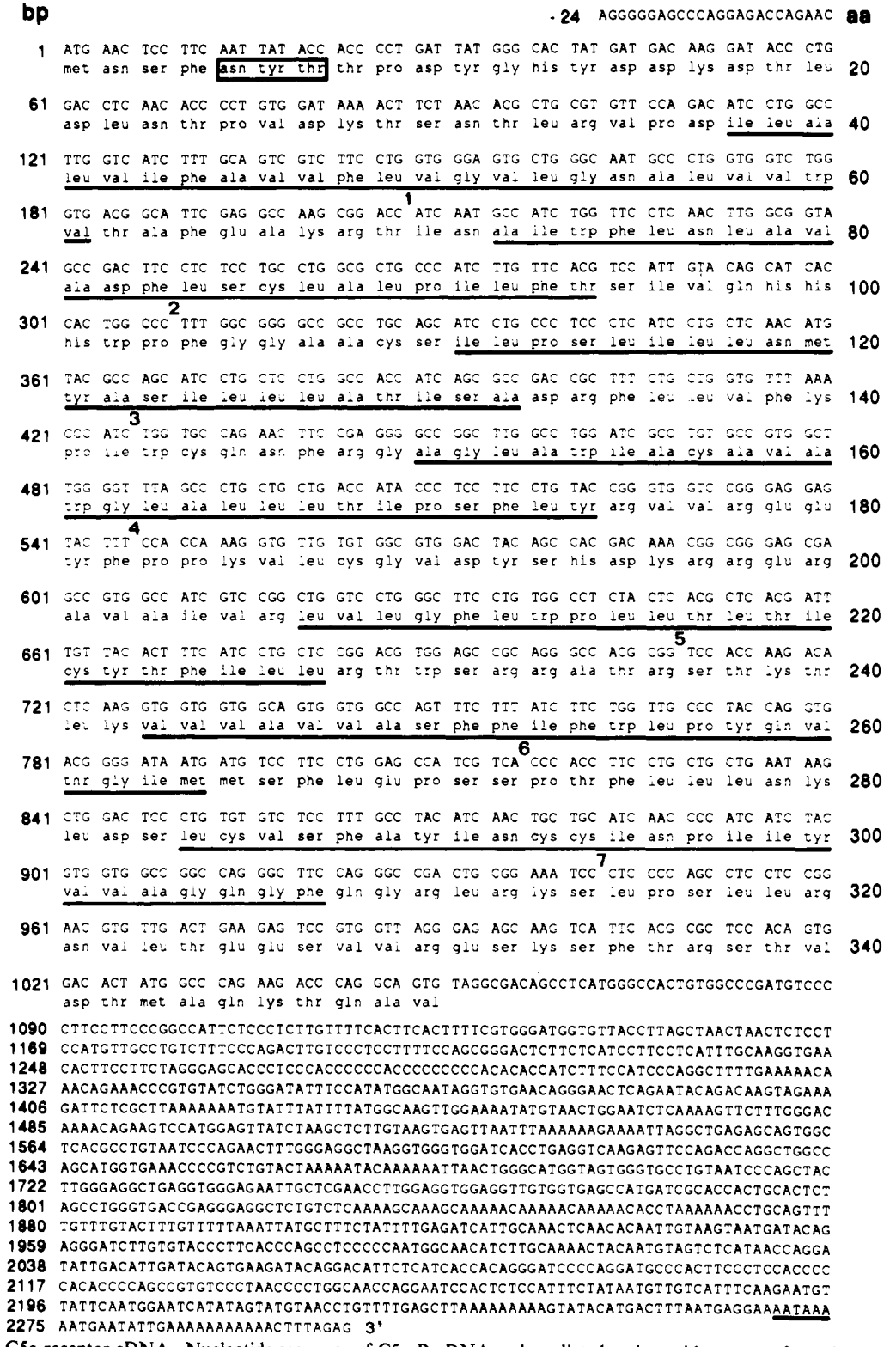

FIGURE 1: Human C5a receptor cDNA. Nucleotide sequence of C5a-R cDNA and predicted amino acid sequence from the translated region. The numbers on the left side indicate nucleotide positions, and numbers on the right side refer to the amino acid sequence. The potential N-glycosylation site (Asn-Tyr-Thr at position 5) is boxed, and the consensus AATAAA polyadenylation signal is underlined. The seven hydrophobic segments predicted by hydropathy plotting according to Kyte and Doolitte (1982) are underlined and numbered. The nucleotide sequence data reported has been submitted to GenBank under accession number J05327.

°C. Furthermore, binding of ^{125}I-hrC5a was abolished by a 500-fold excess of unlabeled hrC5a but not by an excess of *N*-formylpeptide or IL-8 (not shown). The characterization of this C5a-R cDNA clone is fully described hereafter.

cDNA Characterization and Sequence. The insert in the cDNA clone was excised by *Xba*I digestion and analyzed by agarose gel electrophoresis. This insert was 2.3 kb long. Nucleotide sequencing revealed the presence of a short 5′ untranslated region (24 bp), a large open reading frame of 1050 bp, and a 3′ untranslated region extending for an additional set of 1254 nucleotides that contains a potential consensus hexanucleotide (AATAAA) polyadenylation signal at position 2269 (Figure 1). Although the first ATG codon is not in a context that corresponds to the consensus sequence

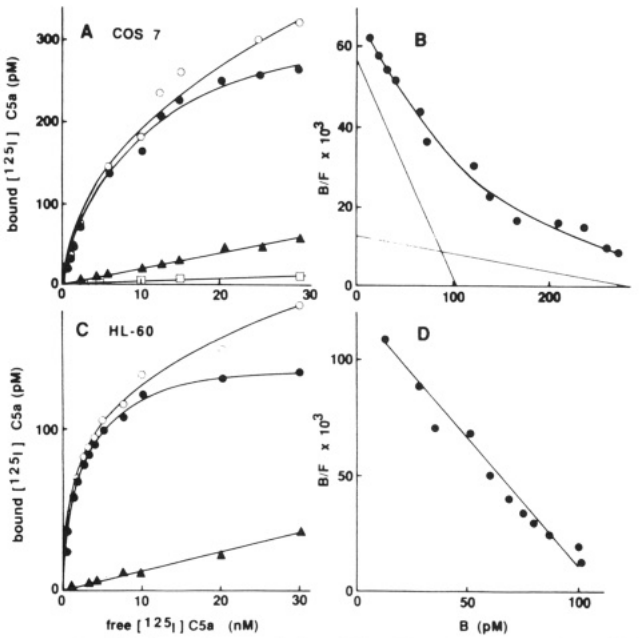

FIGURE 2: Binding characteristics of the C5a-R expressed in COS cells and differentiated HL-60 cells. Saturation isotherms and Scatchard plots with ^{125}I-hrC5a in the presence or absence of 500 nM unlabeled hrC5a, either on COS cell monolayers (3×10^5 cells in a final volume of 1 mL) transfected for 48 h with C5a-R cDNA (A and B) or on HL-60 cells (1×10^6 cells in a final volume of 500 μL) treated with 500 μM Bt2cAMP for 46 h (C and D). Specific binding (●) was determined by subtracting the amount of radioactive ligand bound to the cells in the presence of unlabeled hrC5a (▲) from the amount of radioactive ligand bound in the absence of competitor (○). In panel A, a control experiment indicates that nontransfected COS cells lack the C5a receptor; (□) represents the difference between the radioactivity bound to nontransfected COS cells in the absence or the presence of a 250-fold excess of unlabeled hrC5a. B = bound; B/F = bound/free.

typical of initiation sites of eukaryotic mRNA (Kozak, 1987), we assumed that it could be used as an initiation site, since the isolated C5a-R cDNA encodes a receptor that appears functional as to its expression on the surface of transfected COS cells and its capacity to bind ^{125}I-hrC5a with high affinity. However, the possibility that some of the 5′ end of the transcript is missing cannot be completely ruled out. If the first ATG is assumed to be the initiation site, the predicted translation product is 350 amino acids long, with a calculated M_r of 39 295 Da and a calculated isoelectric point of 9.04. The translated product contains a single potential N-linked glycosylation site in the amino-terminal region (Asn-Tyr-Thr, at position 5). Although there is so far no clear biochemical evidence for the presence of an N-linked oligosaccharide, a glycosylation site is likely in view of the difference observed between the calculated molecular mass (39 kDa) and the apparent molecular mass of 47 kDa observed on sodium dodecyl sulfate gels after labeling with a cleavable C5a photoactivable derivative (Johnson & Chenoweth, 1987).

The hydropathy plotting of the predicted amino acid sequence, according to the method of Kyte and Doolittle (1982), showed that the C5a receptor sequence encodes a polypeptide with seven highly hydrophobic domains (not shown). This pattern is remarkably similar to that of rhodopsin, which is consistent with the C5a receptor being a member of the G-protein-coupled receptor superfamily. The N- and C-terminal regions are predominantly hydrophilic, the N-terminal domain being negatively charged with 7 aspartyl residues for 3 lysyl and arginyl residues. The C-terminal domain is rich in serine and threonine residues, suggesting that the C5a receptor may be phosphorylated.

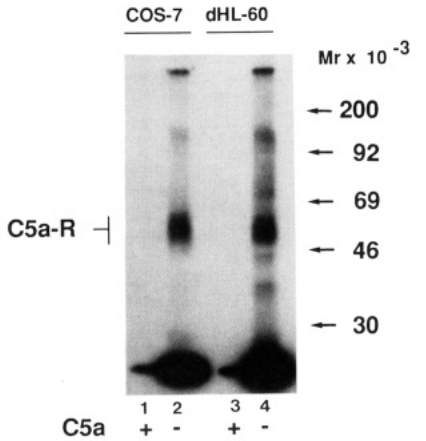

FIGURE 3: Specific cross-linking of ^{125}I-hrC5a to Bt2cAMP-treated HL-60 cells and transfected COS cells. The cross-linking experiments with DSS were carried out as described under Materials and Methods. Approximately 10^6 transfected COS cells (lanes 1 and 2) and 2×10^6 Bt2cAMP-treated HL-60 cells (lanes 3 and 4) were incubated at 4 °C with 10 nM ^{125}I-hrC5a. Cells were allowed to bind the ligand in the presence of 500 nM unlabeled hrC5a (lanes 1 and 3) or its absence (lanes 2 and 4). Gel electrophoresis was carried out on a 10% polyacrylamide Laemmli gel under denaturing and reducing conditions with molecular weight markers (rainbow markers from Amersham). The autoradiograph was exposed for 8 days.

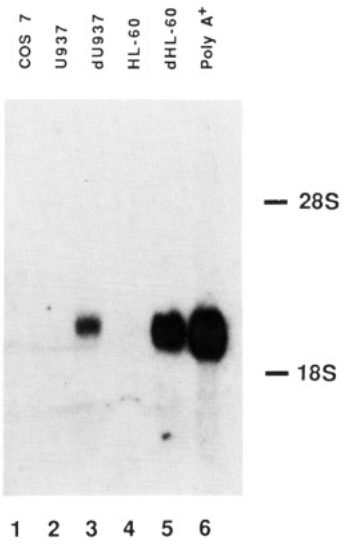

FIGURE 4: Northern blot analysis. Samples of total RNA (10 μg) from COS cells (lane 1) and from U937 and HL-60 cells, either undifferentiated (lanes 2 and 4, respectively) or differentiated for 72 h with 1 mM Bt2cAMP (lanes 3 and 5, respectively), and 1 μg of poly(A+) mRNA from differentiated HL-60 cells (lane 6) were electrophoresed in denaturing formaldehyde/agarose gel and transferred to Hybond-N. RNAs were hybridized to a 0.35-kb ^{32}P-labeled restriction fragment (*Hin*dIII–*Pst*I) of C5a-R cDNA, as indicated under Materials and Methods. The nylon membrane was washed and exposed for 60 h to a 3M X-ray film.

Characterization of the Recombinant C5a Receptor Expressed in COS Cells. To further determine the identity of the isolated cDNA clone with the C5a receptor, we tested whether the binding parameters of ^{125}I-hrC5a to the recombinant C5a receptor expressed in COS cells was similar to those found for the endogenous C5a receptor expressed in differentiated HL-60 cells. Therefore, in three independent experiments, we examined ^{125}I-hrC5a binding at equilibrium, either on differentiated HL-60 cells in suspension or on COS-7 cells adherent to tissue culture plates. The Scatchard transformations of the binding isotherms were analyzed by the graphic method described by Rosenthal (1967). As shown in

FIGURE 5: Sequence similarities. The amino acid sequence of human C5a-R was aligned with that of human *N*-formylpeptide receptor (fMLP-R) (Boulay et al., 1990b) and RDC1 from a dog thyroid (Libert et al., 1989) by using the program ALIGN of the DNA star software. Several residue gaps (-) were introduced to optimize the alignment between the three sequences. The putative transmembrane domains are shaded; their positions were tentatively assigned on the basis of the hydropathy profile and by analogy with other G-protein-coupled receptors. Amino acid similarities are boxed.

Figure 2A, ^{125}I-hrC5a binding to transfected COS cells was not saturable in the range of concentrations (0.1–30 nM) used in these experiments, and the binding isotherm was best fitted with a model with two classes of binding sites. Thus, although the C5a-R cDNA encodes a single polypeptide chain, COS cells transfected with C5a-R cDNA displayed high- and low-affinity binding sites with, on average, 25% of the binding sites as the high-affinity class and 75% as the lower affinity class (K_{d1} = 1.5–2 nM, $n_1 = 2 \times 10^5$ sites/cell; K_{d2} = 20–25 nM, $n_2 = 6 \times 10^5$ sites/cell) as illustrated in Figure 2B by the Scatchard plot analysis. In contrast, ^{125}I-hrC5a binding to Bt2cAMP-differentiated HL-60 cells was rapidly saturable (Figure 2C), and a single class of binding sites with approximately 50 000 sites/cell and an apparent K_d of about 0.8–0.9 nM could be observed (Figure 2D). Similar results have previously been observed and discussed in the case of the *N*-formylpeptide recombinant receptor (Boulay et al., 1990b). The presence of high- and low-affinity binding sites in transfected COS cells most likely results from the interaction of the recombinant C5a receptor with GTP-binding protein(s) present in this cell line. However, additional mechanisms, including posttranslational modifications and/or aggregation of the transiently expressed receptor, may also account for the presence of two classes of binding sites.

Covalent cross-linking of ^{125}I-hrC5a to membrane of transfected COS cells and differentiated HL-60 cells was used to further examine the molecular characteristics of the recombinant C5a receptor. Intact cells, either in suspension or adherent to the plates, were incubated with ^{125}I-C5a in the presence or absence of unlabeled competing ligand at 4 °C. The cells were then washed, incubated with disuccinimido suberate (DSS), and subjected to sodium dodecyl sulfate gel electrophoresis and autoradiography as described under Materials and Methods. As observed by others on human neutrophils and differentiated myeloid U937 cells (Rollins & Springer, 1985; Johnson & Chenoweth, 1985, Gerard et al., 1989), the cross-linked hrC5a–receptor complex migrated as a broad band with an apparent molecular mass of 52–55 kDa in both transfected COS cells and differentiated HL-60 cells (Figure 3, lanes 2 and 4). The labeling was specific, as evidenced by the total lack of radiolabeling when an excess of unlabeled hrC5a was simultaneously added during the binding step (Figure 3, lanes 1 and 3). Thus, if the normal C5a receptor is glycosylated, the recombinant C5a receptor appears to be glycosylated in a similar manner.

Differentiation-Induced Expression of C5a Receptor. As examined by RNA blot analysis, COS cells, undifferentiated U937 cells, and undifferentiated HL-60 cells show no hybridization with the ^{32}P-labeled *Hin*dIII–*Pst*I cDNA fragment (Figure 4, lanes, 1, 2, and 4). The C5a-receptor transcript was identified only in total or poly(A+) RNAs isolated from differentiated myeloid cells. However, although U937 and HL-60 cells were differentiated under the same conditions, i.e., with 1 mM Bt2cAMP for 72 h, the C5a transcript seems to be less abundant in U937 cells than in HL-60 cells (Figure 4, lanes 3 and 5, respectively). Similar results were obtained

when cells were differentiated for 5 days with 1.2% dimethyl sulfoxide (not shown). The full-length transcript of 2.4–2.5 kb is slightly larger than the 2.3-kb cDNA isolated by expression cloning.

Structural Similarities to Other Receptors. In a recent study on the human *N*-formylpeptide receptor (Boulay et al., 1990b), we have hypothesized that receptors of the neutrophil plasma membrane, including receptors for fMLP, C5a, LTB4, IL-8, and PAF, belong to the same subfamily of G-protein-coupled receptors. Furthermore, we have shown that the human *N*-formylpeptide receptor has 24% amino acid identity with RDC1, a putative G-protein-coupled receptor of unknown function isolated from a dog thyroid cDNA library (Libert et al., 1989). This prompted us to align the amino acid sequence of the C5a receptor with that of the *N*-formylpeptide receptor and RDC1. As illustrated in Figure 5, the C5a receptor shows a higher degree of similarity with the fMLP receptor than with RDC1; the percentage of matched residues is 34.2% between C5a-R and fMLP-R in a 304 amino acid overlap and 25.6% between C5a-R and RDC1 in a 281 amino acid overlap.

With the exception of the N-terminal regions, the identities between C5a-R and fMLP-R appear to be distributed throughout the amino acid sequence. While the regions of greatest sequence similarity are concentrated in the hydrophobic segments I, II, IV, VI, and VII with percentages of matched residues of 58%, 48%, 37%, 45%, and 40%, respectively, the percentage of amino acid identity decreased to 22% and 23% in domains III and V. According to the model proposed for the β-adrenergic receptor (Strader et al., 1989), the ligand-binding pocket lies within the hydrophobic transmembrane domains of the receptor, with a contribution from the transmembrane domains III and V in the attachment of the ligand with the receptor. Therefore, it is tempting to speculate that the weak amino acid similarities in hydrophobic domains III and V may partially account for the selective recognition of the *N*-formyl-Met-Leu-Phe tripeptide or the 74 amino acid long C5a anaphylatoxin.

The sequence comparison in the hydrophilic regions of C5a-R and fMLP-R revealed a relatively high degree (37%) of amino acid identity in the first and second extracellular loops (between domains II and III and domains IV and V, respectively), which may be relevant for a similarly folded structure. It has been recently proposed for the rhodopsin model that the extracellular loops have a structural function in the assembly of a functional tertiary structure that is believed to be stabilized by a disulfide bond between cysteine residues 110 and 187 (Doi et al., 1990; Karnik & Khorana, 1990). In this line, it is worth noting that Cys-109 and Cys-188 in C5a-R are invariant throughout fMLP-R, RDC1, and other receptors as well, with the exception of the *mas* oncogene (Young et al., 1986). These cysteine residues may form a disulfide bridge equivalent to the intradiscal disulfide bond in rhodopsin. The third extracellular loop (between domains VI and VII) is very different between C5a-R and fMLP-R, whereas six amino acids out of 17 are identical between C5a-R and RDC1. Although the functional significance of the third loop in the ligand recognition has to be established, this latter area may provide specific and additional sites of interaction with a large ligand such as the C5a anaphylatoxin. In the case of the neuropeptide P receptor, it has been hypothesized that the third extracellular loop is required for a high-affinity interaction with the ligand (Hershey & Krause, 1990).

Since both C5a-R and fMLP-R evoke similar responses in polymorphonuclear neutrophils, they should carry out on their cytoplasmic face similar biochemical and regulatory functions and, therefore, present a relatively high degree of amino acid identity in the cytoplasmic loops and C-terminal regions. In fact, this is partially the case. While the percentage of matched residues is 44% in the second cytoplasmic loop (between domains III and IV), the third cytoplasmic loop (between domains V and VI) that is thought to be critical for the G-protein interaction (O'Dowd et al., 1988) is widely different between C5a-R and fMLP-R as to amino acid composition. However, the third cytoplasmic loop of C5a-R shares a common feature with that of fMLP-R; in both cases, this loop contains a potential phosphorylation site (RSTK or KSSR) for protein kinase A. In addition, with the exception of the *mas* oncogene (Young et al., 1986), this loop is the shortest loop (15 amino acids) found in the G-protein-coupled receptor superfamily. Taken together, these observations suggest that different G proteins of the same subtype, i.e., sensitive to *B. pertussis* toxin, may be used as transducing elements.

In conclusion, the cloning of a second chemoattractant receptor will open the way to discovering other receptors that belong to the same subfamily. Further studies, using domain switching, site-directed mutagenesis, and photolabeling approaches, will be needed to delineate the structural requirements for ligand selectivity as well as the amino acids involved in the ligand binding pocket. The primary structures of C5a-R and fMLP-R being established, it will be of great interest to define whether different G proteins are involved in the transduction pathway when neutrophils are stimulated with either fMLP or C5a.

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The T-arm of tRNA Is a Substrate for tRNA (m^5U54)-Methyltransferase[†,‡]

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ABSTRACT: Fragments of *Escherichia coli* FUra-$tRNA_1^{Val}$ as small as 15 nucleotides form covalent complexes with tRNA (m^5U54)-methyltransferase (RUMT). The sequence essential for binding includes position 52 of the T-stem and the T-loop and extends toward the 3′ acceptor end of FUra-tRNA. The in vitro synthesized 17mer T-arm of *E. coli* $tRNA_1^{Val}$, composed of the seven-base T-loop and 5-base-pair stem, is a good substrate for RUMT. The K_m is decreased 5-fold and k_{cat} is decreased 2-fold compared to the entire tRNA. The T-arm structure could be further reduced to an 11mer containing the loop and two base pairs and still retain activity; the K_m was similar to that of the 17mer T-arm, whereas k_{cat} was decreased an additional 20-fold. The data indicate that the primary specificity determinants for the RUMT-tRNA interaction are contained within the primary and secondary structure of the T-arm of tRNA.

tRNA (m^5U54)-methyltransferase [EC 2.1.1.35, tRNA (uracil-5-)-methyltransferase] catalyzes the methylation by AdoMet of U54 all prokaryotic and most eukaryotic tRNAs. The function of m^5U (ribothymidine) in tRNA remains uncertain, but it appears to improve the fidelity of protein synthesis (Kersten et al., 1981), stabilize the structure of tRNA (Davenloo, et al., 1979), and show a selective advantage in *Escherichia coli* cell growth (Bjork & Neidhardt, 1975). The catalytic mechanism of RUMT[1] is analogous to those of dTMP synthase and the DNA (m^5C)-methyltransferases (Santi & Danenberg, 1984; Santi & Hardy, 1987; Wu & Santi, 1987); it involves initial formation of a covalent Michael adduct between an enzyme nuclophile, probably a Cys thiol, and the 6-position of the target U54 of tRNA to activate the 5-position for subsequent one-carbon transfer. We have reported that, in the presence of AdoMet, RUMT and FUra-tRNA form a covalent complex that can readily be monitored by a gel-shift assay of RUMT on SDS-PAGE (Santi & Hardy, 1987); free RUMT migrates as a 42-kDa protein, whereas the covalent enzyme-tRNA complex migrates as a 65-kDa band (Santi & Hardy, 1987). It has been surmised that in this complex the 6-position of FUra-54 is covalently bound to the enzyme and the 5-position of FUra-54 is methylated.

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[1] Abbreviations: FUra-$tRNA_1^{Val}$, $tRNA_1^{Val}$ containing substitution of Ura by FUra; AdoMet, *S*-adenosylmethionine; Py, pyrimidine; Pu, purine; RUMT, *E. coli* tRNA (m^5U54)-methyltransferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, tlc, thin-layer chromatography.

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