Construction and Expression of a Novel Recombinant Anaphylatoxin, C5a-N19, as a Probe for the Human C5a Receptor†

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ABSTRACT: We have constructed a novel recombinant C5a anaphylatoxin (C5a-N19) containing a 19-residue amino-terminal extension peptide, using a plasmid vector which secretes the nascent polypeptide to the *Escherichia coli* periplasmic space. C5a-N19 was purified from cell lysates by immunoaffinity chromatography using a monoclonal antibody which recognizes a portion of the amino-terminal extension peptide. C5a-N19 was characterized as biologically indistinguishable from the unmodified recombinant anaphylatoxin for release of lysosomal enzymes from dibutyryl-cAMP-differentiated U937 cells. In contrast to unmodified C5a, which is not recognized by anti-C5a antibodies following binding to its cellular receptor, receptor-bound C5a-N19 is recognized by the monoclonal antibody directed against the amino-terminal extension sequence. Because the monoclonal antibody recognizes the C5a–receptor complex on cells, this methodology is useful in fluorescence sorting of C5a receptor-positive cells. A C5a receptor affinity column was constructed by saturating monoclonal antibody bound to agarose with C5a-N19. Digitonin-solubilized C5a receptor from dibutyryl-cAMP-induced U937 cells was adsorbed to the matrix and eluted by dissociation of the ligand–receptor complex from the antibody. Analysis by SDS–polyacrylamide gel electrophoresis revealed a unique protein band at 41K, consistent with the molecular weight predicted from cross-linking experiments when the contribution of C5a is subtracted. Development of this recombinant C5a derivative provides a useful probe previously unavailable for the C5a receptor molecule.

The complement anaphylatoxin peptide C5a participates in the inflammatory response through a number of biological pathways. The anaphylatoxin causes the directed migration (chemotaxis) of inflammatory cells (Ward & Newman, 1969). C5a can metabolically activate inflammatory cells to release toxic oxygen species ("respiratory burst") and cause the exocytosis of catabolic enzymes from intracellular granules (Henson, 1971; Goetzl & Austen, 1974). In addition to these effects on inflammatory cells, the C5a molecule has been shown to enhance vascular permeability and cause contraction of smooth muscle containing tissues (Bernauer et al., 1972; Hugli & Muller-Eberhard, 1978; Stimler et al., 1980; Marceau & Hugli, 1984). Finally, the anaphylatoxin can amplify the inflammatory response through the stimulation of cytokine production (Goodman et al., 1982; Okusawa et al., 1988).

At present, a limited amount of information is available on the cellular receptor(s) for C5a. The most characterized system is the human polymorphonuclear neutrophil (PMN),[1] where several groups have identified a unique receptor (Johnson & Chenoweth, 1985a; Rollins & Springer, 1985; Huey & Hugli, 1985). The plasma membrane of the PMN possesses $\sim 10^5$ high-affinity binding sites per cell ($K_d \sim 1$ nM). Chemical cross-linking of radioiodinated C5a on this cell type using a variety of chemical and photochemical reagents yields identical results, a single band with an apparent molecular weight of 52–55K on SDS–PAGE.[1] When the solubilized receptor is studied, an additional species with an apparent molecular weight of 95K has been observed (Rollins et al., 1988). These data have been interpreted as the cross-linking of another molecule, possibly a GTP-binding protein, to the C5a–receptor complex.

Further biochemical analysis of the receptor protein has been hampered by limitations imposed by the C5a molecule itself. A variety of structure–activity studies have been performed with the native and recombinant molecules which may be summarized as follows. In contrast to the C3a anaphylatoxin where short carboxyl-terminal peptides mimic and equal the activity of the native molecule (Hugli & Erickson, 1977; Gerary-Schahn, 1988), the biological activity of C5a is dependant on a tertiary structure which presents elements of the amino-terminal region, the disulfide-linked core, and the carboxyl-terminal pentapeptide (Chenoweth et al., 1979; Gerard et al., 1979, 1985; Johnson & Chenoweth, 1985b). Because of these structure–activity features, chemical modification of the C5a molecule often leads to loss of receptor binding. For example, iodination or nitration at tyrosine-23 reduces receptor affinity (Johnson & Chenoweth, 1985b), as does reaction at lysyl and arginyl residues with group-specific reagents. The α-amino group is accessible to reaction without dimunition of activity (van Epps & Chenoweth, 1984; Himmelfarb et al., 1990), but the yield of such modifications is poor, and the reaction products representing the spectrum of reactive amino groups are difficult to separate. Because of these features, the potential for analyzing the C5a receptor

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; cAMP, adenosine cyclic 3′,5′-phosphate; PMN, polymorphonuclear; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid.

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using C5a as the probe has been limited. Further, as predicted from the structure–activity relationships described, attachment of C5a to an insoluble matrix predictably leads to loss of receptor-binding activity (unpublished observations).

An alternative approach could involve the use of antibodies against C5a to purify ligand–receptor complexes. Unfortunately, in all instances tested, such antisera do not recognize C5a in association with its receptor.

An added difficulty in characterizing C5a receptors stems from the problems in dealing with the PMN, first because it is difficult to obtain very large numbers of these cells on a routine basis ($\sim 10^{10}$ are required for nanomolar quantities of receptor). Second, the large degradative capacity of these cells often results in extensive proteolysis of solubilized receptor (Gerard et al., 1989). An alternative strategy involves the use of the malignant cell lines HL-60 and U937, which are capable of expressing receptors for the chemoattractants, the formyl oligopeptides, and the complement peptides, when cultured in the presence of the appropriate growth or differentiation factors (Harris & Ralph, 1985; Chenoweth & Soderberg, 1985). Previous work by Chenoweth and Soderberg demonstrated high-affinity binding of iodinated C5a to U937 cells cultured in the presence of membrane-permeable cAMP analogue dibutyryl-cAMP (Chenoweth & Soderberg, 1985; Johnson & Chenoweth, 1985a). These data suggested the presence of C5a receptors on this cell type analogous to those found on PMN's, based on the apparent molecular weight of cross-linked ligand–receptor complexes.

Recently, monoclonal antisera recognizing the amino acid sequence Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (Flag sequence) have become available as a method for immunoaffinity purification and identification of recombinant molecules (Hopp et al., 1988; Prickett et al., 1989). Introduction of the DNA encoding this short hydrophilic sequence upstream of the coding sequence in the plasmid for a recombinant molecule thus provides a tailored monoclonal recognition site. This amino acid sequence constitutes a natural substrate for enterokinase and, if desired, can be selectively removed by enzymatic treatment of the recombinant molecule following immunoaffinity purification. Our knowledge of the structure–activity relationships for the C5a molecule suggested the possibility of engineering a recombinant peptide containing this addition amino-terminal sequence, separated from the "natural" amino terminus of C5a by a spacer sequence of 11 residues, generating the new recombinant C5a-N19. Such a molecule would potentially provide a reagent which could simultaneously be recognized by both the cellular receptor and the monoclonal antibody. In addition to providing a "tag" for cell-sorting or immunohistochemical studies, C5a-N19 could theoretically be selectively derivatized to a column support via the monoclonal antibody to serve as an affinity column for purification of the C5a receptor.

In this paper, we report the development of a novel C5a-N19 recombinant molecule and demonstrate the utility of this reagent in the analysis and isolation of the anaphylatoxin receptors on the U937 cell line.

Experimental Procedures

Construction of C5a-N19. The construction of the expression plasmid pC5a has been previously described (Franke et al., 1988). The plasmid was purified from *E. coli* GE196 cells by alkaline lysis followed by CsCl density gradient centrifugation (Birnboim & Doly, 1979), and 10 μg was double-digested with *Eco*RI and *Sal*I (Boehringer Mannheim), liberating the entire coding sequence for native C5a. The expression vector PpL7,[2] containing the λ promoter and the thermolabile c1857 λ phage repressor (2 μg), was double-digested with the aforementioned restriction enzymes, and the products of both digests were purified by agarose gel electrophoresis. DNA was detected by ethidium bromide staining, and the appropriate bands were cut from the gel under brief UV transillumination. The DNA was purified by using GeneClean (Bio101, La Jolla, CA), and vector and insert were ligated to one another by using T4 DNA ligase (5 units) (New England Biolabs) at 13 °C for 18 h. The ligation mixture was transformed into competent *E. coli* N4830-1, and transformants were grown at 30 °C on LB plates containing 50 μg/mL ampicillin.

Expression and Purification of Recombinant C5a and C5aN19. Recombinant C5a containing the native 74-residue sequence with an additional N-terminal methionyl residue was purified as previously described (Franke et al., 1988). This recombinant is produced as an insoluble inclusion body in *E. coli* GE196, and must be thoroughly reduced and denatured in guanidine hydrochloride containing 2-mercaptoethanol prior to renaturation and purification by column chromatography and reverse-phase HPLC. For the purification of C5a-N19, ampicillin-resistant transformant colonies were grown at 30 °C in SIM medium (20 g/L yeast extract, 32 g/L tryptone, M9 salts, 0.1 mM $MgSO_4$, 0.001 mM $FeCl_3$, 0.2% glucose, and 0.1 mM $CaCl_2$) containing 50 μg/mL ampicillin to an absorbance of 0.5 A_{550} units/mL with constant agitation. At that time, the cells were heat-shocked by the addition of one-third volume of medium at 60 °C, producing a rapid shift in the culture temperature to 42 °C. The cells were allowed to grow for an additional 6–18 h at 42 °C, recovered by centrifugation at 4200*g* for 15 min, and stored as a frozen cell paste at −20 °C. The recombinant fusion protein was recovered by using a modification of a protocol described previously (Hopp et al., 1988). Briefly, the cell pellets obtained from approximately 500 mL of culture were thawed and suspended in 50 mM sodium phosphate, pH 8.4, containing 0.15 M sodium chloride, 0.25 mg/mL lysozyme, and 5 mM PMSF. The suspension was frozen (−70 °C) and thawed (37 °C) 3 times, $CaCl_2$ was added to a final concentration of 1 mM, and lysis was completed by sonicating. The resulting *E. coli* lysate was centrifuged at 25000*g* for 45 min at 4 °C, and the supernatant was applied to a 1-mL column of monoclonal antibody coupled to an agarose support (Anti-Flag, Immunex, Seattle, WA), equilibrated in 50 mM sodium phosphate, pH 7.4, containing 0.15 M sodium chloride (PBS) which was made 1 mM in $CaCl_2$ within 24 h of use. Following washing of the column, the bound material was eluted by the addition of 2 mM EDTA. The monoclonal antibody has an absolute requirement for calcium to bind the amino-terminal extension sequence of the peptide produced (Prickett et al., 1989), and addition of EDTA leads to the desorption of specifically bound material. The material recovered from the affinity gel was dialyzed versus 5% acetic acid and lyophilized. Final purification of recombinant protein was accomplished by using reverse-phase HPLC as previously described (Gerard et al., 1985). C5a-N19 was analyzed by SDS–polyacrylamide gel electrophoresis as described by Laemmli (1970) and silver-stained (Bio-Rad) or electroblotted onto PVDF membranes (Matsudaira, 1987) (Immobilon, Millipore) and detected by using either monoclonal antibody against the amino terminus or polyclonal rabbit anti-human C5a, horseradish peroxidase

[2] The PpL7 expression vector containing the ompA signal sequence, the Flag sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), and a polylinker region was obtained through a license granted by Immunex Corp., Seattle, WA.

labeled goat anti-rabbit IgG (Sigma), and finally 2,2′-diaminobenzidine in the presence of H_2O_2 (Towbin et al., 1979).

Chemical Characterization of Recombinant C5a. Recombinant C5a-N19 was analyzed by amino acid analyses of samples hydrolyzed in HCl vapor at 150 °C for 2 h using a Beckman Model 5000 instrument. The amino acid sequence of the recombinant was confirmed by using an Applied Biosystems gas-phase sequencer.

Cell Culture. U937 cells were obtained from the American Type Culture Collection and were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics as described (Sundstrom & Nilsson, 1976). Cells were grown to a density of (1–2) × 10^6/mL and subcultured by diluting 1:4 with fresh media. Induction of C5a receptors was accomplished by adding dibutyryl-cAMP (Sigma) at 0.5–1 mM to the cells 1 day after subculture; culture expansion and cAMP stimulation thus occurred simultaneously.

Bioassays. U937 cells were assayed for the release of the lysosomal enzyme *N*-acetyl-β-D-glucosaminidase as previously described (Franke et al., 1988). Cells were suspended in Hank's balanced salt solution (HBSS) (Gibco) containing 10 mM HEPES, pH 7.4, at 6.4 × 10^6/mL and equilibrated at 37 °C for 20 min. C5a dilutions and cytochalasin B (5 μL of 1 mM solution in DMSO) were combined in glass test tubes and incubated for 1 min at 37 °C before addition of 0.5 mL of cells. After 3-min incubation, the samples were transferred to ice/water and centrifuged at 400*g* for 3 min at 4 °C. The supernatants were assayed for released *N*-acetyl-β-D-glucosaminidase in duplicate by mixing 0.2 mL with 0.2 mL of 8 mM *p*-nitrophenyl-*N*-acetyl-β-glucosaminide (Sigma) in 42.5 mM sodium acetate, pH 4.5, and incubating for 30 min at 37 °C. Reactions were quenched by the addition of 0.4 mL of 0.4 M glycine–NaOH, pH 10.4, and the absorbance at 405 nm was determined. The maximum enzyme release was determined by using 0.2% Triton X-100, and the absorbance at 405 nm for this sample was the same as that observed for the highest C5a or C5a-N19 concentrations tested.

Receptor Binding Assays. The C5a receptor was assayed by using ^{125}I-C5a labeled as previously described (Gerard et al., 1989). Briefly, recombinant C5a (rC5a) was incubated in 0.2 M sodium phosphate, pH 7.2, with immobilized glucose oxidase and lactoperoxidase (Enzymobeads, Bio-Rad), 0.3% β-D-glucose, and sodium [^{125}I]iodide (New England Nuclear) for 25 min at room temperature. The reaction mixture was centrifuged to remove enzymes, and free ^{125}I was removed by gel filtration on a 6-mL column of Bio-Gel P6-DG (Bio-Rad) in PBS, pH 7.4, containing 0.1% bovine serum albumin (BSA). The specific activity routinely obtained with this protocol is ~50 Ci/mmol.

Binding to either whole cells or solubilized receptor was performed at 4 °C in Hank's balanced salt solution (HBSS), containing 25 mM HEPES, pH 7.4, 0.25% BSA, and 0.1 mM PMSF. Following incubation with intact cells (1 × 10^6 in 0.5 mL) or solubilized receptor preparations, the bound ligand was separated from free by filtration using glass fiber filters (Whatman GF/F) pretreated with 0.1% poly(ethylenimine). In the case of solubilized receptor, the binding mixture in a volume of 100 μL was mixed with 250 μL of 0.1% bovine γ-globulin and 250 μL of 30% poly(ethylene glycol). The mixture was vortexed and allowed to precipitate on ice for 5 min before transfer to the filtration manifold. The glass fiber filters were washed rapidly with 3 × 10 mL aliquots of ice-cold 5 mM potassium phosphate, pH 7.4, containing 0.27 M sucrose for intact cells, or 3 × 3 mL of 10% PEG for solubilized receptor preparations. The filters were dried and assayed by γ counting. Controls for nonspecific binding were performed under identical conditions in the presence of 1 μM unlabeled rC5a.

Fluorescence-Activated Cell Sorting. Dibutyryl-cAMP-induced U937 cells were suspended in duplicate in microcentrifuge tubes in 50 μL of PBS, pH 7.4, containing 5% fetal bovine serum and 0.2% NaN_3 (binding buffer) at 2.5 × 10^7 cells/mL. Cells were incubated either alone, with 50 nM C5a-N19, or with 1 μM native rC5a and 50 nM C5a-N19, to control for nonspecific binding, for 15 min on ice, collected, and washed via brief centrifugation. They were next incubated in 50 μL of binding buffer with monoclonal antibody (2 μg) for 15 min on ice. Cells were washed as described above, and the resulting pellets were resuspended in the original volume of binding buffer, mixed with 12.5 μg of FITC-conjugated goat anti-mouse IgG (Becton Dickinson), and allowed to stand on ice for a final 15 min. Unbound FITC-labeled secondary antibody was removed by repeated centrifugation and resuspension in binding buffer, and cell proteins were fixed by resuspension in 1 mL of 1% paraformaldehyde in PBS. The processed samples were analyzed by using a Becton-Dickinson FACStar sorter.

Detergent Solubilization of U937 Cells. Following 3–4 days in culture with 1 mM dibutyryl-cAMP, U937 cells [(3–5) × 10^9] were collected by centrifugation at 1500*g* for 10 min at 4 °C. All subsequent procedures were performed at 4 °C. The cell pellet was resuspended in an equal volume of phosphate-buffered saline (PBS) containing 0.2 mM phenylmethanesulfonyl fluoride (PMSF) and 20 μg/mL each of soybean trypsin inhibitor, leupeptin, and chymostatin. The suspension was then mixed with an equal volume of PBS containing 2% digitonin (Wako) and incubated on ice for 15 min with occasional mixing. This mixture was centrifuged at 75000*g* for 20 min and the supernatant used for binding experiments or affinity purification of the C5a receptor as described below. Protein concentration was determined by using a Coomassie blue based assay (Pierce) standardized with BSA.

Affinity Chromatography of the C5a Receptor Using C5aN19 Monoclonal Antibody Columns. A C5a-N19 affinity column was prepared by loading 40 μg of C5a-N19 on 1 mL of immobilized monoclonal antibody resin in PBS containing 1 mM $CaCl_2$ (~2.5 mg of purified antibody/mL of gel). The supernatant solution obtained from digitonin solubilization of (3–5) × 10^9 cAMP-induced U937 cells (~15 mL) was diluted to a final volume of 50 mL with PBS containing 0.25 mM PMSF. $CaCl_2$ was added to a final concentration of 1 mM. This solution was applied to the column of C5a-N19-saturated monoclonal antibody at a flow rate of 2 mL/h at 4 °C. Following a wash with 50 column volumes of PBS containing 1 mM $CaCl_2$, 0.1% digitonin, and 0.25 mM PMSF, C5a-N19 and the C5a receptor were eluted with 10 mL of PBS containing 5 mM EDTA, 0.1% digitonin, and 0.25 mM PMSF by adding 1-mL aliquots at 30-min intervals. Aliquots of the final 10 mL of wash buffer and the EDTA eluate were labeled with ^{125}I Bolton–Hunter reagent (New England Nuclear) for 20 min at 0 °C as described (Bolton & Hunter, 1973). Excess reagent was consumed by the addition of an equal volume of Tris-containing SDS sample buffer with 2-mercaptoethanol. Samples were heat-denatured and applied to a 15% SDS–polyacrylamide gel. Following electrophoresis, gels were soaked in 40% methanol/10% acetic acid to remove free Bolton–Hunter reagent, dried, and autoradiographed at −70 °C (Kodak X-OMAT AR-5). The remainder of each pool was concentrated by lyophilization, redissolved in 0.5 mL of water, and precipitated with TCA–tRNA (Peterson, 1973).

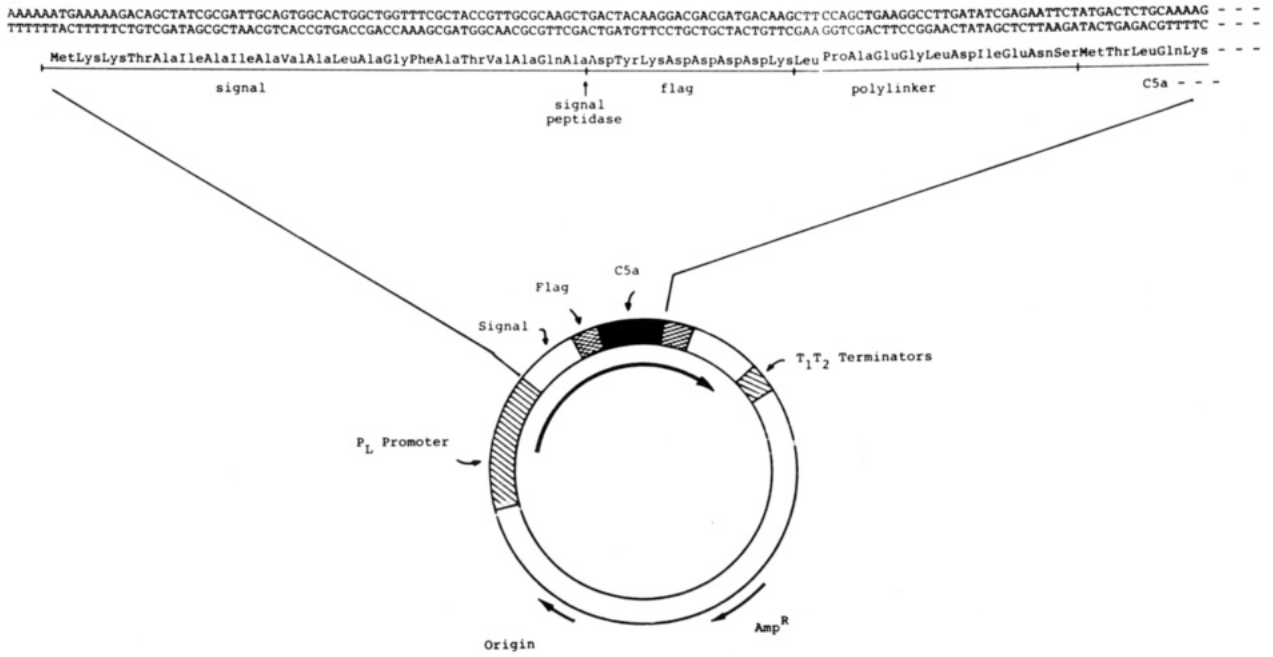

FIGURE 1: Schematic diagram of the expression plasmid pC5a-N19.

The pellets obtained were dissolved in a small volume of 0.1 M Tris base, diluted with an equal volume of SDS sample buffer containing 2-mercaptoethanol, heated 5 min at 100 °C, and applied to a 15% SDS–polyacrylamide gel. Following electrophoresis, the gel was electroblotted to PVDF membranes (Immobilon, Millipore) as described by Matsudaira (1987) at 180 mA for 1 h at 22 °C using a semidry apparatus (JKL, Denmark). The blot was stained with Coomassie blue dye as described previously.

RESULTS

Construction and Analysis of C5a-N19. In order to further characterize the U937 cell C5a receptor, a novel recombinant anaphylatoxin molecule was constructed. The properties of the modified peptide were designed to provide a reagent more amenable to chemical and immunological manipulation. The synthetic C5a gene previously characterized (Franke et al., 1988) was spliced into the expression vector pPL7 in order to synthesize the fusion protein depicted in Figure 1. The ompA signal peptide sequence utilized in pPL7 directs the nascent recombinant protein to the periplasmic space, where it is processed to the mature recombinant in soluble form, consisting of the 8-residue monoclonal recognition sequence and an 11-residue spacer. The expression vector uses the λ PL promoter, which can be controlled by the thermolabile repressor product of the Cl locus, contained in the chromosome of the host strain N4830-1. In this system, no potentially toxic recombinant protein is produced during culture at 30 °C, while cells are grown to mid-log phase. Rapid temperature shift to 42 °C results in inactivation of the repressor and allows protein synthesis from the PL promoter. When aliquots of the cultures were analyzed by SDS–PAGE followed by Western blotting with the antibody against the amino-terminal sequence, a signal was detected as early as 4 h following heat-shock of the culture, and was stable for at least 18 h (not shown).

On the basis of these preliminary observations, cultures of *E. coli* N4830-1 containing the recombinant expression vector were grown for 12 h at 42 °C, and the cells were lysed to release proteins from the periplasmic space. The soluble cell extract was applied to an affinity column containing the monoclonal antibody against the amino terminus cross-linked to agarose, and washed with PBS containing calcium. Since this antibody binds to the amino-terminal sequence with an absolute requirement for calcium (Prickett et al., 1989), chelation of calcium by EDTA in the elution buffer resulted in selective desorption of the desired product. SDS–PAGE analysis of the immunoaffinity purification is shown in Figure 2. Western blotting of the column fractions using the anti-amino-terminal antibody shows a single species of expressed recombinant with an apparent molecular weight of ~12K. The same band was stained by an antibody against human C5a (not shown). Silver staining of gels of the same column fractions indicates the presence of minor contaminants which are subsequently eliminated by reverse-phase HPLC (not shown).

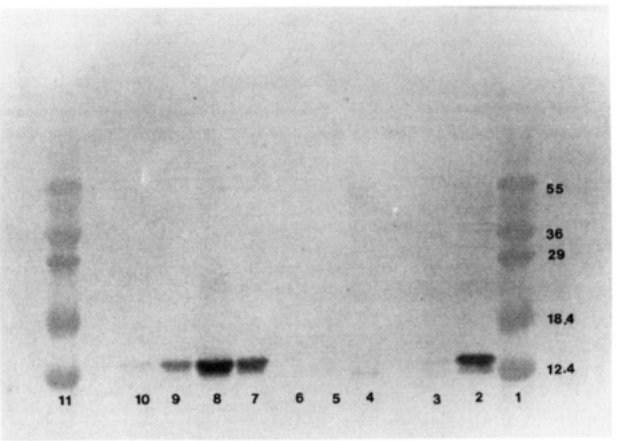

FIGURE 2: Western blot analysis of immunoaffinity purification of C5a-N19. Lanes 1 and 11, molecular weight standards ($\times 10^{-3}$); lane 2, *E. coli* N4830-1 supernatant after lysis; lane 3, cell pellet; lane 4, immunoaffinity column runthrough; lane 5, wash; lanes 6–10, EDTA-eluted fractions 1–5, respectively.

Amino acid analysis of the purified product indicated a yield of approximately 250 μg of recombinant C5a-N19 per liter of *E. coli* culture under these conditions. In order to prove that the expressed material was synthesized with the correct sequence, a 250-pmol aliquot was subjected to gas-phase microsequencing. Twenty-two cycles of Edman degradation established the sequence predicted by the plasmid construct (Figure 1).

Table I[a]

preparation	total protein (mg)	total binding act.	sp act. (pmol/mg of protein)	stepwise yield (%)
intact cells	351 (4.9 × 10^6 cells)	1.95 ± 0.7 pmol	5.22 (398 × 140 fmol/10^6 cells)	
digitonin solubilized	220 × 20	590 ± 123 pmol	2.68 ± 0.56	30
affinity column unretained	~220 ± 20	232 ± 52 pmol	1.06 ± 0.24	(39)
affinity column retained	ND[b]	358 pmol	ND	61
affinity column eluate	~0.05	22.5 pmol	450	10

[a] Summary of the affinity purification of the C5a receptor from dibutyryl-cAMP-induced U937 cells. Results are the average of eight independent preparations. [b] ND, not determined.

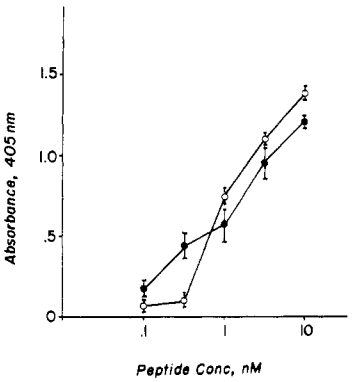

FIGURE 3: Release of *N*-acetyl-β-D-glucosaminidase from U937 cells cultured 72 h in 1 mM dibutyryl-cAMP, by C5a-N19 and C5a. Cells were incubated with C5a-N19 (○) or rC5a (●) and cytochalasin B in the concentrations shown. Released *N*-acetyl-β-D-glucosaminidase was determined as described under Experimental Procedures. Maximal release, as elicited by 0.2% Triton X-100, resulted in an absorbance at 405 nm of 1.49 ± 0.12. All points determined in duplicate.

Biological Characterization of C5a-N19. In order to demonstrate that the C5a-N19 recombinant is capable of binding to and transducing signals via the C5a receptor, dibutyryl-cAMP-induced U937 cells were analyzed for release of the granule enzyme *N*-acetyl-β-D-glucosaminidase. Cytochalasin B treated cells were incubated with varying concentrations of either C5a-N19 or the recombinant product based on the native C5a structure. As shown in Figure 3, C5a-N19 was indistinguishable from the "natural" sequence, with an ED_{50} of 1–2 nM. An identical result was also obtained when human peripheral PMN neutrophils were examined for the C5a-mediated release of the granule enzyme myeloperoxidase (data not shown).

As further evidence that the two recombinants recognize the same receptor, their ability to compete with radiolabeled C5a for binding to the receptor was tested. Cells were incubated with ^{125}I-C5a in the presence of 0–200 nM unlabeled rC5a or C5a-N19. As shown by the data in Figure 4, the two recombinants were equipotent for competing with the specific binding of radiolabeled C5a.

Fluorescent Cell Sorting of C5a Receptor Positive U937 Cells. Since the C5a-N19 recombinant was capable of recognizing the C5a receptor on cAMP-induced U937 cells, the ability of the anti-amino-terminal antibody to identify receptor-bound C5a-N19 was examined. Cells were incubated with 50 nM C5a-N19 alone or with 1 μM rC5a to control for nonspecific binding. The histograms obtained from FACS analysis are displayed in Figure 5. Comparison of the results obtained with 50 nM C5a-N19 alone indicate that approximately 68% of the dibutyryl-cAMP-induced U937 cells are specifically recognized by the anti-amino-terminal antibody when C5a-N19 is bound to their surface. When excess rC5a was included in the incubation to block specific binding of C5a-N19, the histogram was shifted back to the position of the antibody control.

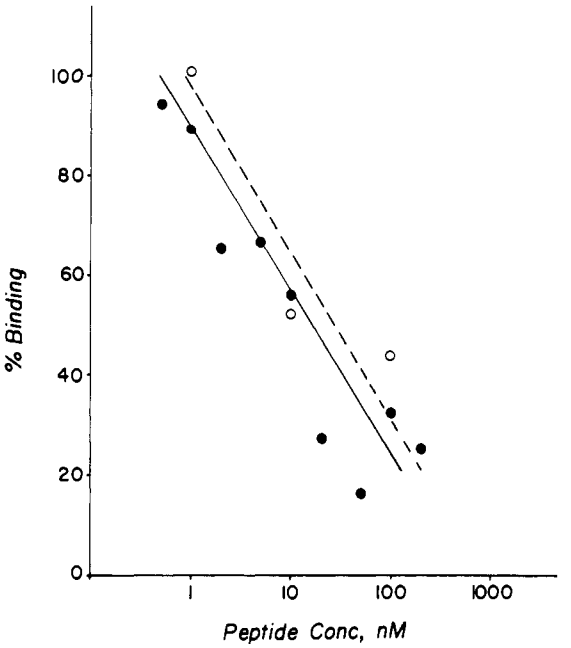

FIGURE 4: Competition for binding of ^{125}I-C5a by C5a-N19 (○) or rC5a (●). Unlabeled ligands were added in increasing concentrations to mixtures containing 1 nM ^{125}I-C5a and U937 cells (2 × 10^6/mL) and incubated under equilibrium conditions at 4 °C. Percent of binding is calculated as the percent of ^{125}I-C5a bound in the presence and absence of unlabeled ligand after subtraction of the ^{125}I-C5a bound in the presence of 1 μM unlabeled C5a.

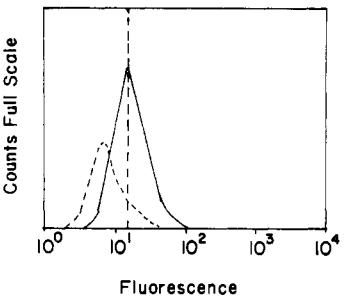

FIGURE 5: Binding of FITC-labeled anti-mouse IgG to cells incubated with C5a-N19 (50 nM) and the anti-amino-terminal antibody (solid line) or with rC5a (1 μM), C5a-N19 (50 nM), and the anti-amino-terminal antibody (dashed line). Cells incubated with FITC-labeled antibody alone gave a histogram superimposable on the nonspecific binding curve (dashed line).

Affinity Chromatography of the U937 Cell C5a Receptor. We next examined the ability of immobilized C5a-N19–anti-amino-terminal antibody complex to act as an affinity ligand for the solubilized C5a receptor. Previous work by Rollins et al. (1988) demonstrated the usefulness of digitonin in liberating a functional C5a receptor from human PMN membranes. We confirmed these findings with dibutyryl-cAMP-induced U937 cells. Digitonin (1%) was effective in solubilizing ~30–50% of the total receptor present based on Scatchard analyses of the solubilized cell supernatant (Table I). The affinity of this material was slightly lower than that

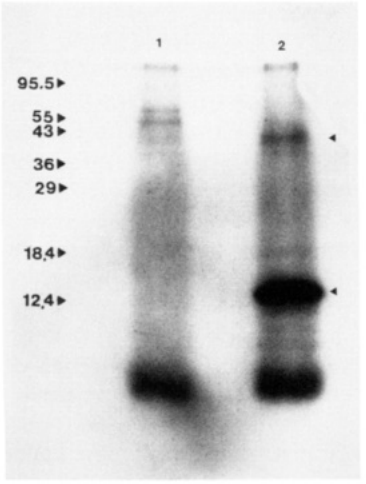

FIGURE 6: Representative autoradiogram obtained after SDS–PAGE of ^{125}I Bolton–Hunter-labeled fractions collected from the C5a receptor affinity column (C5a-N19–anti-amino-terminal antibody) just before (lane 1) and after (lane 2) elution with EDTA. Molecular weight markers shown × 10^{-3}. Arrows in lane 2 indicate the position of distinct proteins eluting with EDTA at ~41 and at 12–13 kDa (C5a-N19).

measured on intact cells (2–5 nM compared with ~1 nM on intact cells). Solubilized receptor remained fully active for up to 4 days at 4 °C. Rapid loss of activity occurred thereafter, concomitant with an increase in the turbidity of the solution, presumably reflecting instability of the digitonin solution.

The solubilized receptor was diluted with PBS to a final concentration of 0.3% digitonin, and $CaCl_2$ was added to a final concentration of 1 mM. This preparation was applied to a column containing 40 μg of C5a-N19 attached to the anti-amino-terminal antibody affinity gel (column volume 1 mL). In a series of experiments, we observed that the column runthrough contained approximately 40% of the original solubilized receptor binding activity. Reapplication of this fraction to a second, fresh column resulted in no further adsorption of material to the column. Binding analysis of this "unbindable" fraction consistently revealed a small but significant increase in the affinity constant from 1–2 nM compared with 2–5 nM for the total solubilized fraction.

Following extensive washing, the column was eluted by chelating the calcium in the buffer with EDTA. The fractions immediately preceding and following the EDTA elution were analyzed by SDS–PAGE after labeling with ^{125}I Bolton–Hunter reagent. Representative results are presented in Figure 6. Comparison of the column eluates before and after the addition of EDTA reveals a unique protein at ~41 kDa. The C5a-N19, which is also eluted by EDTA, is evident at 12–13 kDa.

Since the material eluting from the column is complexed with C5a-N19, it is not possible to precisely determine the total amount of receptor activity present in the eluate. When freed from excess C5a-N19 by chromatography with Bio-Gel A 0.5M in buffer containing 0.25 M NaCl, the excluded protein fraction is capable of specific binding to ^{125}I-C5a and indicates a yield of approximately 5%. However, significant amounts of C5a-N19 remain in this fraction, and thus quantitation of the amount of receptor present is a minimum one.

Discussion

The present study was undertaken with the primary objective of developing a C5a analogue which is a more versatile probe for its receptor than the natural molecule. We have previously shown that a synthetic C5a gene can be expressed in *E. coli* as an insoluble protein, which can be denatured, refolded, and purified to yield biologically active anaphylatoxin (Franke et al., 1988). A commercially available monoclonal antibody which recognizes the amino-terminal sequence of a unique octapeptide can be used in the purification and analysis of recombinant proteins, providing the peptide is spliced at the amino terminus of the recombinant. This monoclonal antibody demonstrates calcium-dependent binding to the first three residues of the octapeptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (Flag sequence) (Prickett et al., 1989). In the case of C5a, no data were available concerning the consequences of the addition of peptide sequence to the amino-terminal region, although other modifications can be made at this position without altering biological activity (van Epps & Chenoweth, 1984; Himmelfarb et al., 1990). We reasoned that if a peptide sequence could be added to the C5a amino terminus, the optimum situation would involve placing the monoclonal site some distance from the natural sequence by a "spacer peptide" in order to minimize steric constraints. Using the expression vector pPL7, we made a construct which places the Flag sequence 11 residues removed from the amino-terminal methionine of recombinant C5a. An additional feature of this construct is the placement of the ompA signal sequence on the nascent polypeptide; this sequence directs the recombinant to the periplasm where it is cleaved, leaving soluble recombinant protein (Figure 1).

In preliminary experiments, the expression of a polypeptide with the expected mobility of C5a-N19 was detected by Western immunoblot of transformed N4830-1 cells only in response to heat-shock (Figure 2). This result indicated that the precursor for C5a-N19 (Figure 1) was properly processed by the cells, since the monoclonal requires a free amino-terminal aspartate residue for effective binding (Prickett et al., 1989). Thus, in contrast to our earlier construct, which produces an insoluble protein inclusion in *E. coli* GE196 (Franke et al., 1988), the present recombinant was secreted and processed in the periplasm as expected. The preparative production of C5a-N19 was conveniently performed using 500–2000-mL quantities of transformed cells, grown to mid-log phase at 30 °C, heat-shocked, and maintained at 42 °C for 12 h. When the supernatant fraction from the cell lysate was adsorbed to and eluted from the immunoaffinity column, SDS–PAGE and Western immunoblot analysis, using either the anti-amino-terminal monoclonal antibody (Figure 2) or polyclonal anti-C5a, revealed a single protein with the predicted mobility of the recombinant anaphylatoxin. While only a single band was observed under these conditions, amino acid analysis of the preparations did not reveal the expected stoichiometry for the recombinant, suggesting the presence of impurities. Reverse-phase HPLC analysis of the immunoaffinity eluate revealed a second molecule, which was not further characterized; the major product contained the exact composition and amino-terminal sequence of C5a-N19. The yield of C5a-N19 averaged ~250 μg/L of *E. coli*, roughly comparable to the amount of material obtained by using our previous construct.

These results indicated that the C5a-N19 molecule was expressed and processed as a soluble product and thus was likely spontaneously folded. Since six of the seven cysteine residues of C5a form three intrachain disulfide bonds which are essential for biological activity (Gerard et al., 1979), the simplest test for proper folding of the anaphylatoxin measures the specific activity of the recombinant as a secretagogue for lysosomal enzymes from U937 cells or PMNs. Since C5a-N19 was equipotent to native recombinant C5a for stimulating the

release of U937 granule-bound *N*-acetyl-β-D-glucosaminidase (Figure 3), we conclude that the addition of the 19-residue extension peptide does not interfere with either the correct spontaneous folding of the anaphylatoxin or the binding of the fusion protein to the cellular receptor in a biologically meaningful way. Further evidence for the latter conclusion is supported by the observation that both recombinants are equivalent in competing for specific binding of ^{125}I-C5a on cAMP-treated U937 cells (Figure 4).

Since C5a-N19 is capable of binding to the U937 cell C5a receptor, we next examined the ability of the monoclonal antibody directed at the amino-terminal Flag sequence to recognize the cell-bound ligand. Approximately 68% of the cells were positive for binding of the monoclonal antibody as demonstrated by fluorescence-activated cell sorting (Figure 5). These results are in excellent agreement with those reported by Chenoweth and Soderberg (1985), who found that approximately the same proportion of dibutyryl-cAMP-induced U937 cells bound FITC-conjugated C5a. We conclude that the amino-terminal extension peptide provides, for the first time, an epitope which is not sterically hindered upon binding of C5a to its cellular receptor.

These results demonstrate the feasibility of using a tailored immunoaffinity matrix for the purification of the C5a receptor complex. In contrast, in experiments not detailed here, we have found that the attachment of unmodified C5a directly to column supports results in the biological inactivation of >99% of the peptide. These data are explicable in terms of structure–activity studies which show that the binding of C5a to its receptor requires both conformational features (i.e., intact disulfide bonds) and also specific residues such as tyrosine-23 and the charge effects of lysyl and arginyl side chains. The presence of the "spacer peptide" between the monoclonal epitope and the C5a molecule proper was expected to allow the ligand to maximally interact with the solubilized receptor. As shown in Figure 6 and Table I, we observed that approximately 60% of the digitonin-solubilized C5a receptor was retained on the C5a-N19–anti-amino-terminal antibody column. Curiously, the fraction which was not bound was not retained on a second, fresh column preparation. These data suggest that this fraction contains receptor in a form which is unable to interact with immobilized anaphylatoxin, but can still bind ligand in the fluid phase. Also noted was a slight but reproducible increase in the affinity of the unretained fraction, compared with the solubilized receptor prior to affinity chromatography. One possibility to account for these data is that the affinity column is capable of retaining the C5a binding subunit alone, but for steric reasons cannot bind the complex formed by the receptor with its associated G-protein. The unretained complex of the C5a receptor and the G-protein might be expected to bind the C5a with a higher affinity than the C5a receptor binding subunit alone. Indeed, it has previously been suggested that >20% of the solubilized C5a receptor is complexed with a 43-kDa polypeptide, putatively a specific G-protein, detectable by chemical cross-linking (Rollins et al., 1988).

Material bound to the C5a-N19–anti-amino-terminal antibody column was gently eluted using EDTA and was analyzed by SDS–PAGE following radiolabeling with ^{125}I Bolton–Hunter reagent. The pattern reproducibly observed demonstrated the presence of a protein with an apparent molecular weight of 41K, as predicted for the receptor when the contribution of C5a to the cross-linked complex is subtracted. Varying amounts of polypeptides with apparent molecular weights of 38K and 24K were also observed in some preparations, which presumably reflects some degradation of the binding moiety during isolation and/or concentration. The main disadvantage of this methodology is that the C5a-N19 which coelutes with the receptor interferes with subsequent binding studies, and is difficult to remove completely by gel filtration without resorting to denaturing conditions.

It is of interest to note by comparison that the affinity matrix designed by Regan et al. (1986), for purification of the α_2-adrenergic receptor from human platelets, provided a similar degree of purification (~100-fold) and recovery (~15% overall, after the first affinity step).

In sum, we have shown that a peptide sequence containing a characterized monoclonal antibody epitope can be spliced at the amino terminus of the C5a molecule, which can be expressed as a soluble, biologically active recombinant, C5a-N19. The addition of 19 N-terminal amino acids at the N-terminus of C5a does not appear to affect the spontaneous folding or biological potency of the molecule, as judged by receptor binding and biological activity. The monoclonal antibody recognizes receptor-bound C5a-N19, and can be used to "tag" C5a receptor-positive cells. Finally, the monoclonal antibody can be used as an immunoaffinity matrix for purification of solubilized C5a receptors. This approach provides a new tool in the study of C5a and its cellular receptors, and may be broadly applicable to the study of other biologically active peptides or proteins.

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Isolation of a Terminal Cisterna Protein Which May Link the Dihydropyridine Receptor to the Junctional Foot Protein in Skeletal Muscle†

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ABSTRACT: The isolated dihydropyridine receptor and junctional foot protein were employed as protein ligands in overlay experiments to investigate the mode of interaction of these two proteins. As previously demonstrated by Brandt et al. [Brandt et al. (1990) *J. Membr. Biol. 113*, 237–251], the DHP receptor directly binds to an intrinsic terminal cisterna protein of M_r 95 000 (95-kDa protein). The junctional foot protein also binds to an M_r 95 000 protein showing similar organelle distribution to the 95-kDa protein which binds to the dihydropyridine receptor. The 95-kDa protein which binds to the dihydropyridine receptor was isolated to over 85% purity employing sequential column chromatography. Junctional foot protein and dihydropyridine receptor overlays of the column fractions at successive stages of isolation show an identical pattern of distribution, indicating that both probes bind to the same protein. When CHAPS-solubilized terminal cisterna/triads were passed through Sepharose with attached 95-kDa protein, the junctional foot protein was specifically retained, as evidenced by ryanodine binding. The junctional foot protein was incompletely released by 1 M NaCl. The α_1 subunit but not the β subunit of the dihydropyridine receptor was also specifically retained, as evidenced by immunoblotting employing dihydropyridine receptor subunit-specific antibodies. A 170-kDa Stains-all blue staining protein, which appears to be bound to the luminal side of the terminal cisterna, was also retained on the 95-kDa protein column. From these findings, a model for the triad junction is proposed.

One major hypothesis for excitation–contraction (EC) coupling, as originally proposed by Schneider and Chandler (1973), states that the voltage sensor molecules in the transverse (T)-tubule undergo a conformational change that is transmitted through the feet of the terminal cisternae (TC) abutting the T-tubule to activate the sarcoplasmic reticulum (SR) Ca^{2+} release. The junctional foot protein (JFP) was originally identified as a high molecular weight doublet by Cadwell and Caswell (1982) and later isolated and localized to the triad junction by immunoelectron microscopy (Kawamoto et al., 1986). Subsequent biochemical and electrophysiological studies indicated that the JFP is the ryanodine-sensitive Ca^{2+} release channel of the SR (Imagawa et al., 1987; Hymel et al., 1987; Lai et al., 1988). The dihydropyridine (DHP) receptor is present at unusually high density in the T-tubules (Fosset et al., 1983; Glossman et al., 1983), but only a few percent of the DHP receptor appear to function as Ca^{2+} channels (Schwartz et al., 1985). Rios and Brum (1987) have proposed that the DHP receptor is the voltage sensor of the T-tubule. Tanabe et al. (1988) have supported this proposal in studies of dysgenic mice which exhibit EC coupling only after introduction of cDNA for the α_1 subunit of the DHP receptor. On the basis of these findings, several models have been proposed in which the DHP receptor and JFP bind directly to each other. Conformational changes of

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