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Functional high efficiency expression of cloned leukocyte chemoattractant receptor cDNAs

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Human kidney 293 TSA cells were transfected by a calcium phosphate method with human formylpeptide and C5a receptor cDNAs with high efficiency. Formylpeptide receptor positive transfectants expressed a total of 968,000 ± 34,000 receptors per cell with two affinity states (K_ds of ca. 0.43 nM and 39 nM), which in the presence of 100 μM GTPγS decreased by ca. 4-fold the number of high-affinity sites. The ligand binding pharmacology of cloned and expressed formylpeptide receptors were indistinguishable from endogenous receptors on human neutrophils. Expressed formylpeptide and C5a receptors were functionally active in mobilizing intracellular calcium via a pertussis toxin sensitive mechanism with an ED_{50} for formylpeptide of ca. 0.5–1.0 nM. This expression system, in which receptor expression can be monitored by flow cytometric methods and in which intracellular calcium responses are measurable, unlike in the more popular COS-7 cell expression system, will provide a useful basis for the analysis of chemoattractant receptor structure–function relationships.

Formylpeptide; Chemoattractant receptor; Gene expression; C5a

1. INTRODUCTION

The ability of phagocytic leukocytes (polymorphonuclear leukocytes and macrophages) to accumulate and become activated at sites of inflammatory reactions is a prerequisite for their role in host defense. The normal functioning of phagocytes in inflammation requires the coordinate regulation of migratory vs. cytotoxic activities. Phagocytes migrate along gradients of chemoattractants produced by humoral and cellular immune responses. At higher concentrations, chemoattractants have the ability to stimulate the cells' cytotoxic responses. Defined chemotactic factors include C5a, a complement proteolytic fragment, leukotriene B4 (LTB_4), an arachidonate metabolite, platelet-activating factor (PAF), certain products of bacterial protein synthesis (i.e. formylmethionyl peptides) and a family of chemotactic cytokines including interleukin-8 (reviewed in [1,2]).

The best characterized chemotactic factor receptor is the one which binds formylpeptides. While much is known of how this receptor initiates cellular activation [1,2], the precise mechanisms by which chemoattractant receptors initiate migratory versus cytotoxic activities remains to be determined.

The availability of cloned cDNAs encoding chemoattractant receptors will provide tools for genetic approaches to determining their structure–function relationships and means of receptor desensitization. To this end we report the development of a highly efficient transient cDNA expression system in which cloned and expressed chemoattractant receptors are functionally competent to evoke ligand-mediated intracellular calcium mobilization. In addition, the binding pharmacology and the ability to couple to pertussis toxin sensitive G proteins mimics endogenous chemoattractant receptors on human neutrophils.

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2. MATERIALS AND METHODS

2.1. *Materials*

PMA, norepinephrine, carbachol, C5a, fMet-Met-Met, fMet-Phe, Met-Leu-Phe, fMet, and fluorescein isothiocyanate (FITC) from Sigma. fMet-Leu-Phe and fNle-Leu-Phe-Nle-Tyr-Lys from Peninsula Laboratories (Burlingame, CA). FITC-fMet-Leu-Phe and Indo-1 AM from Molecular Probes Inc. (Eugene, OR). Adenosine triphosphate (ATP) from Pharmacia/LKB. [^{3}H]FMLP (53.6 Ci/mmol) and α[^{32}P]NAD from New England Nuclear. Pertussis toxin from List Biological Laboratories (Campbell, CA). Tissue culture media from Gibco.

2.2. *Cells/cell culture/transfection*

Adenovirus type 5 transformed human embryonic kidney 293 cells from American Type Culture Collection (ATCC no. CRL 1573). TSA cells (a clonal variant of 293 cells stably expressing viral large T antigen) kindly provided by Dr G. Rice, Genentech Inc. Cells were maintained in DMEM/F12 (50:50) supplemented with 10% fetal bovine serum, 2 mM glutamine and containing penicillin/strepto-

mycin. Calcium phosphate mediated transfection of cells was carried out as described [3] using 8–9 μg of CsCl-purified plasmid DNA as a calcium phosphate precipitate per 60 mm plate. Cells were exposed to DNA for 18 h, glycerol shocked for 15 s and analyzed 48 h later. Human neutrophils were isolated from peripheral blood of normal volunteers as described [4].

2.3. *Cytosolic calcium measurements*

Forty-eight hours after glycerol shocking transfected cells were removed from cell culture dishes with Versene (Gibco), washed once with 10 mM HEPES-buffered Hanks balanced salt solution (HHBSS) at room temperature and resuspended in 1.2 ml HHBSS (~5–6 × 10⁶/ml). Cells were loaded with 1 μM Indo-1 AM for 20 min at room temperature, washed twice with HHBSS, resuspended in 1.2 ml HHBSS and placed in a cuvette. The cuvette was placed into a heated (37°C) cuvette holder of a Perkin-Elmer fluorescence spectrophotometer (Model 650-19). Calcium analyses were carried out after equilibration of the cells to 37°C (5 min) with an excitation wavelength of 335 nm and an emission wavelength of 405 nm. Maximal and minimal fluorescence were determined in the presence of 0.02% digitonin and 20 mM Tris-HCl pH 8, 5 mM EGTA respectively. Intracellular calcium levels were measured using the following formula: $[Ca^{2+}]_i = K_d (F-F_{min}/F_{max}-F)$ [5].

2.4. *DNA cloning/plasmid constructions*

Reverse transcription PCR of differentiated HL-60 cell mRNA to obtain formylpeptide receptor encoding cDNA was carried out essentially as described [6] using antisense primer (5′-CTTTGCCTGTAACGCCACCTC-3′) and amplified with antisense and sense primer (5′-ATGGAGACAAATTCCTCTCTCC-3′) using VENT polymerase (New England Biolabs) for 29 cycles (denaturation at 94°C, 30 s, annealing at 55°C, 30 s and extension at 72°C, 30 s). The resulting cDNA fragment was labeled by nick translation and used to screen a λgt10 HL-60d library [7] under low stringency (6 × SSC, 0.25% non-fat dry milk at 42°C) with washing in 2 × SSC, 0.1% SDS at 48°C. Formylpeptide receptor clones were confirmed by double stranded DNA sequencing as described [8]. The coding region of the formylpeptide receptor cDNA from a receptor positive clone was generated by PCR to contain a unique *Eco*RI site immediately 5′ of the start codon and a *Hin*dIII site immediately 3′ to the stop codon using the cloning primers described above which had an additional 12 bases at their 5′ end encoding *Eco*RI and *Hin*dIII sites respectively. The *Eco*RI/*Hin*dIII digested PCR-generated receptor cDNA was then directionally cloned into *Eco*RI/*Hin*dIII cut pRK5 plasmid DNA [9]. A 2.1 kb *Eco*RI-digested cDNA fragment (25 bp of 5′-untranslated sequence and ca. 1.0 kb of 3′-untranslated sequence) encoding the C5a chemoattractant receptor [10] was inserted into the same pRK5 vector.

2.5. *Preparation of membranes*

Membranes from chemoattractant receptor transfected TSA cells were prepared as described for neutrophil membranes [4].

2.6. *ADP-ribosylation of membranes*

Pertussis toxin catalyzed ADP-ribosylation of cell membranes was carried out as described [11] using 50 μg of membrane protein in a reaction mixture containing 0.1% Lubrol, 10 μM NAD, α[^{32}P]NAD (5,000 cpm/pmol) without guanine nucleotides for 30 min at 30°C.

2.7. *Binding assays*

Flow cytometric analysis of FMLP or C5a binding to transfected cells was carried out using 10 nM FITC-FMLP and 5 nM FITC-C5a prepared as described [12]. Cells were incubated with fluoresceinated ligands in HHBSS for 2 h at 4°C, washed and analyzed on an Epics 753 flow cytometer. Radioligand binding of [^{3}H]FMLP to intact cells was carried out on 200 μl cell aliquots (2 × 10⁶ cells) in HHBSS at 4°C for 2 h. Cells were collected by vacuum filtration on Whatman GFC filters, washed 5 times with cold HHBSS and dried filters counted in scintillation fluid. Radioligand binding to membranes was carried out as described above except using 10 mM HEPES pH 7.2, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT as buffer and incubating for 1 h at room temperature [13]. Binding parameters were analyzed by Scatfit computer analysis using a nonlinear least squares curve fitting [14].

3. RESULTS

3.1. *Cloning and expression of formylpeptide receptor cDNA*

cDNA clones encoding the formylpeptide receptor reported by Boulay et al. [15] were isolated from a λgt10 library constructed from mRNA of dibutyryl cyclic AMP differentiated HL-60 cells (HL-60d). Screening was carried out using oligonucleotide primers (24-mer) complementary to the first 8 amino acids (5′ primer) and COOH-terminal 8 amino acids (3′ primer) of the reported receptor sequence [15] were used in a reverse transcription polymerase chain reaction (RT-PCR) with mRNA of HL-60d cells as a template. The resulting cDNA fragment was used to screen the library under low stringency. Receptor clones were selected, from secondary screens using end-labeled 3′ primer under high stringency. Four of nine clones were identical to the previously reported formylpeptide receptor cDNA sequence [15]. The coding region from one of these four clones was transiently expressed in TSA human embryonic kidney cells by calcium phosphate mediated

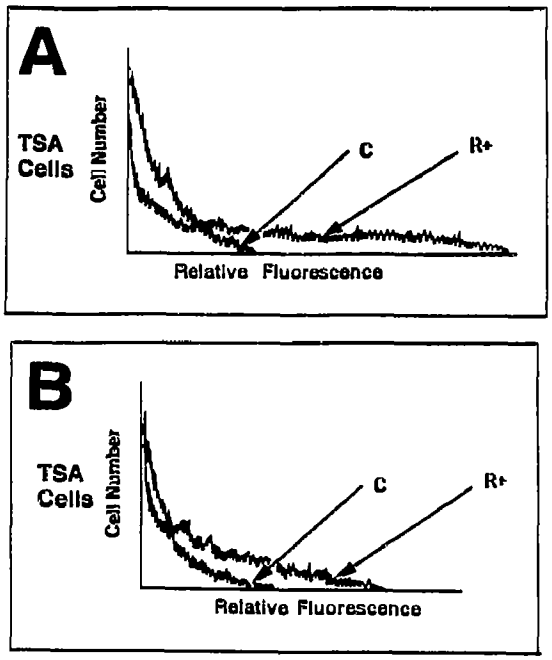

Fig. 1. Analysis by flow cytometry of formylpeptide and C5a receptor expression on transfected cells. Human kidney TSA cells were transfected with formylpeptide receptor cDNA (R+) or plasmid vector alone (C) (panel A) and with C5a receptor cDNA (panel B). Forty-eight hours post-transfection cells, were incubated with 10 nM of fluorescein-conjugated fNle-Leu-Phe-Nle-Tyr-Lys or 5 nM fluorescein-conjugated C5a at 4°C for 2 h. Cells were analyzed by FACS as described in section 2. In the above representative fluorescence patterns, 44% of formylpeptide receptor transfected TSA cells and 35% of C5a receptor transfected cells are expressing receptor.

transfection. Transfection efficiency was measured by flow cytometry using FITC-fNle-Leu-Phe-Nle-Tyr-Lys. Approximately 40–55% of transfected cells expressed formylpeptide receptors (Fig. 1A).

3.2. *Expression of C5a receptor cDNA*

The *Eco*RI cDNA fragment encoding the C5a chemoattractant receptor [10], inserted into the pRK5 vector, was transfected into TSA cells in the same manner as the cloned formylpeptide receptor cDNA. Flow cytometry measurements using FITC-C5a indicated approximately 35–40% of transfected cells expressing C5a receptors (Fig. 1B).

3.3. *Binding specificity of cloned and expressed formylpeptide receptors: comparison with endogenous neutrophil formylpeptide receptors*

The ability of various *N*-formylated peptides to compete with [^{3}H]FMLP binding to expressed formylpeptide receptors was compared with endogenous formylpeptide receptors on human neutrophils (Fig. 2). The order of potency for inhibition of [^{3}H]FMLP binding to expressed formylpeptide receptors was fNle-Leu-Phe-Nle-Tyr-Lys > fMet-Leu-Phe > fMet-Met-Met > fMet-Phe > Met-Leu-Phe > fMet (Fig. 2A). This binding specificity profile was indistinguishable from that observed for the native receptor on human neutrophils (Fig. 2B).

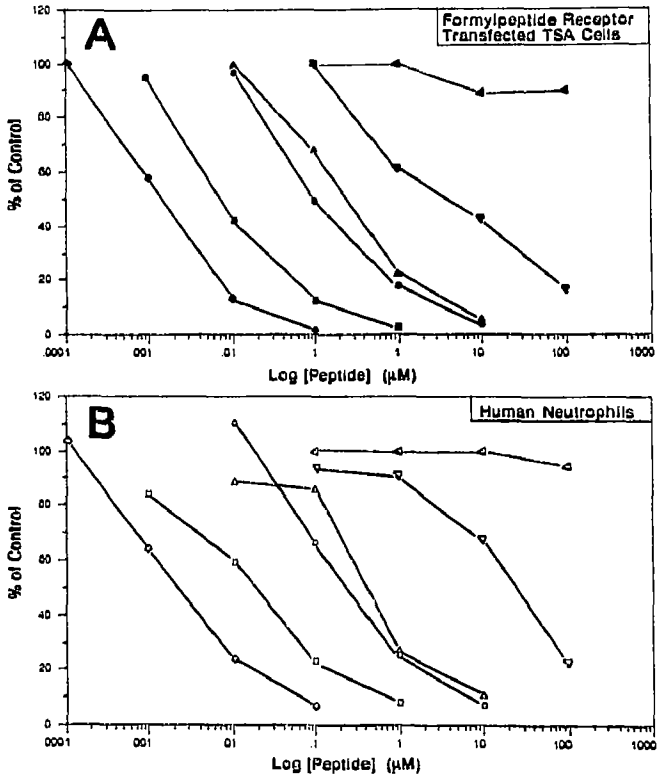

Fig. 2. Comparison of the specificity of [^{3}H]FMLP binding to formylpeptide receptors on human neutrophils vs. receptor transfected TSA cells. Formylpeptide receptor transfected TSA cells (panel A) and human neutrophils (panel B) were incubated with 5 nM [^{3}H]FMLP in the presence of various concentrations of fMet-Leu-Phe (□), fNle-Leu-Phe-Nle-Tyr-Lys (◇), fMet-Met-Met (○), fMet-Phe (△), Met-Leu-Phe (▽) and fMet (◁). Specific binding was calculated and expressed as the percent of [^{3}H]FMLP binding in the absence of any competing peptide. Each value represents the mean of triplicate determinations.

3.4. *Effect of guanine nucleotide on equilibrium binding of FMLP to formylpeptide receptor transfected cell membranes*

Membranes from formylpeptide receptor transfected TSA cells were incubated with [^{3}H]FMLP and specific binding plotted by Scatchard analysis [16]. The total receptor concentration was ca. 2.3 pmol/mg of membrane protein which corresponded to 968,000 ± 34,000 receptors per receptor expressing cell based on flow cytometric measurements of transfection efficiency (data not shown). Membrane preparations of formylpeptide receptor transfected TSA cells showed two affinity states with K_ds of 0.43 ± 0.11 nM for the high-affinity class and 39 ± 7 nM for the low affinity class of binding sites (Fig. 3). Binding of [^{3}H]FMLP in the presence of 100 μM GTPγS decreased by ca. 4-fold the number of high-affinity sites with no effect on total receptor number (Fig. 3).

3.5. *Functionality of expressed formylpeptide and C5a receptors*

The ability of expressed formylpeptide and C5a receptors to transduce ligand-mediated signals was assayed by examining intracellular calcium mobilization. Initial attempts to demonstrate functional transfection in COS cells were unsuccessful even though high efficiency expression was observed (ca. 30% efficiency, data not shown). Using transfected human kidney TSA

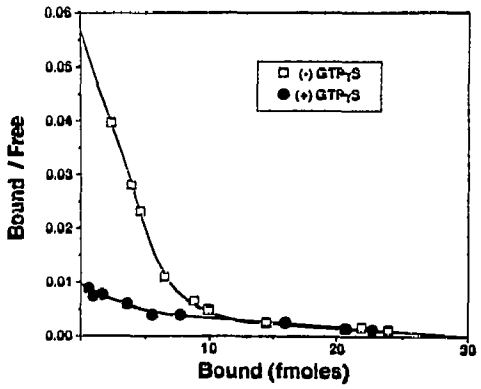

Fig. 3. Scatchard analysis of specific binding to formylpeptide receptor transfected TSA cell membranes. [^{3}H]FMLP was incubated at various concentrations for 1 h at 25°C with 13 μg of formylpeptide receptor-transfected TSA cell membranes per point. Non-specific binding was determined in the presence of 50 μM unlabeled FMLP and subtracted from total binding to determine specific binding. Each point is the mean of triplicate determinations. A two-site model was determined by ScatFit computer analysis. Binding was carried out in the absence (□) or presence (●) of 100 μM GTPγS.

cells a calcium response to FMLP was evident and occurred with a peak at ca. 10 s. The maximum increase was ca. 100 nM above the basal levels of ca. 150 nM. Treatment with 0.5 μg/ml pertussis toxin for 4 h inhibited by ca. 70% the calcium response to FMLP (Fig. 4A). The expressed C5a receptor mobilized intracellular calcium with maximal increases of ca. 100 nM that was also inhibited by ca. 70% by pertussis toxin pretreatment (Fig. 4B). Non-transfected cells showed no calcium response to either FMLP or C5a (data not shown). The specificity of pertussis toxin was indicated by the inability of the toxin to affect the response of TSA cells to norepinephrine, an α1-adrenergic agonist whose receptor utilizes a pertussis toxin insensitive G protein to effect PI hydrolysis and consequent calcium mobilization [17] (Fig. 4C). The extent of ribosylation by pertussis toxin in TSA cells was determined by incubating membranes isolated from cells pretreated with buffer (Fig. 4D, lane 1) or toxin under the above conditions (Fig. 4D, lane 2) with pertussis toxin. The extent of ADP-ribosylation of ca. 40–41 kDa product(s) was greater than 98% as determined by densitometric scanning of the autoradiograph.

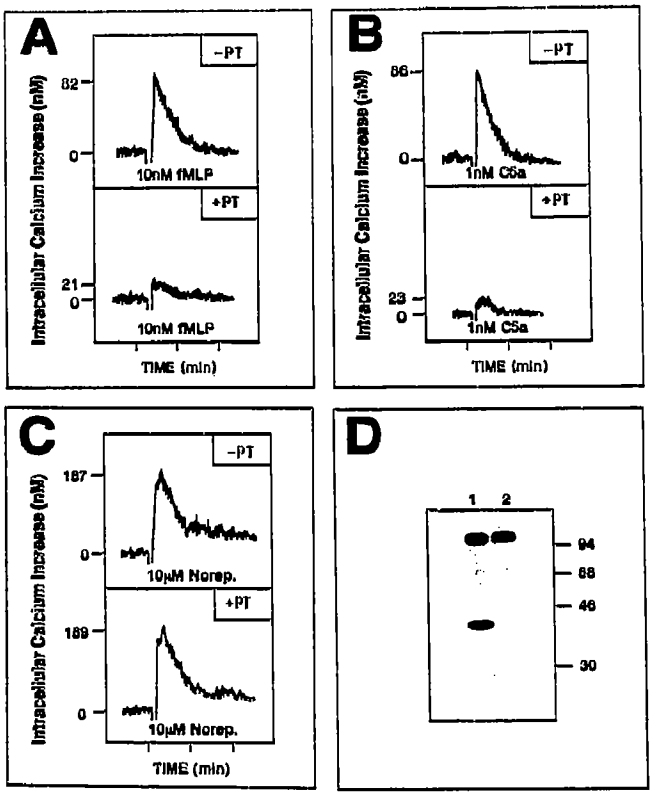

Fig. 4. Analysis of intracellular calcium mobilization in formylpeptide and C5a receptor transfected TSA cells. (A) Formylpeptide receptor transfected cells were incubated in the presence (+PT) or absence (−PT) of 0.5 μg/ml pertussis toxin for 4 h at 37°C and subsequently loaded with Indo-1. Cells were then exposed to 10 nM FMLP and $[Ca^{2+}]_i$ measured. (B) As in panel A with C5a receptor transfected TSA cells exposed to 1 nM C5a. (C) As in panel A using non-transfected TSA cells exposed to 10 μM norepinephrine (Norep.). (D) ADP-ribosylation of TSA cell membranes from control (lane 1) and pertussis toxin pretreated (0.5 μg/ml, 4 h at 37°C) cells (lane 2) by pertussis toxin (15 μg/ml). Membranes (50 μg) were incubated as described in section 2. An autoradiogram of labelled proteins after SDS-PAGE is presented, molecular weight of size standards in kDa.

4. DISCUSSION

A transient expression system in which leukocyte chemoattractant receptors can be expressed with high efficiency and with the ability to initiate biochemical signals will allow the rapid analysis of structural receptor mutants to delineate structure–function relationships. Using calcium phosphate-mediated transfection of formylpeptide and C5a receptor cDNAs we obtained high transfection efficiencies in human embryonic kidney cells. To assess the structural integrity of cloned and expressed chemoattractant receptors we analyzed the binding pharmacology of the formylpeptide receptor in two ways: (1) by determining the specificity of [^{3}H]FMLP binding to receptor transfected cells in parallel with measurements to human neutrophils and (2) by Scatchard analysis of [^{3}H]FMLP binding to formylpeptide receptor transfected cells in the presence or absence of added guanine nucleotide. The specificity of [^{3}H]FMLP binding to formylpeptide receptor transfectants was indistinguishable from human neutrophils with the rank order of potency of various *N*-formylated peptides to compete with FMLP binding being the same. Equilibrium binding experiments carried out with membranes of formylpeptide receptor transfected TSA cells showed two affinity states with K_ds of ca. 0.4 nM and 39 nM with ca. 25% of the receptors in the high affinity state. This compares well with previous binding analyses of formylpeptide receptors on human neutrophil membranes (K_ds of ca. 0.5 and 20 nM with 25% of the receptors being in the high-affinity state [18]). As in neutrophils [13], in the presence of guanine nucleotides there was a ca. 4-fold reduction in high affinity receptors measured on membranes of TSA transfectants with no effect on total receptor number. These data suggested a role for G proteins in the regulation of formylpeptide receptor affinity [19] and indicate a similar degree of G protein association with transfected and expressed formylpeptide receptors on TSA cells as compared to formylpeptide receptors on neutrophils.

The functionality of cloned and expressed formylpeptide and C5a receptors was assessed by measuring the ability of FMLP and C5a to mobilize intracellular calcium in receptor transfected cells. The expressed formylpeptide receptor was capable of mobilizing intracellular calcium in a dose dependent manner (data not shown). The ED_{50} was ca. 0.5–1.0 nM which compares well with the ED_{50} found with human neutrophils (ca. 1 nM [20]). As in the neutrophil, the expressed formylpeptide and C5a receptors are apparently coupled to a pertussis toxin sensitive G protein as evidenced by the ability of pertussis toxin to inhibit by ca. 70% the

calcium responsiveness of cells exposed to FMLP or C5a (Fig. 4A and B). While greater than 98% of the available pertussis toxin substrate(s) was modified by the toxin (Fig. 4D), formylpeptide and C5a receptor transfectants still mobilized intracellular calcium, albeit at ca. 30% of untreated cells. In pertussis toxin treated human neutrophils, a similar degree of ribosylation resulted in a ca. 90% inhibition of calcium mobilization in response to 10 nM FMLP [20]. This observation suggests two possibilities: (1) that only very small amounts of G protein are necessary to elicit a calcium response through association with transfected chemoattractant receptors. Given the extremely high level of receptor expression (ca. 970,000 in transfectants vs. ca. 50,000 in neutrophils [13]) this explanation is certainly plausible; (2) that formylpeptide and C5a receptors are also capable of interacting with a pertussis toxin-insensitive G protein(s) to mobilize calcium.

The ability to transiently express both formylpeptide and C5a chemoattractant receptors with high efficiency in a responsive human cell expression system will now allow for the precise delineation of chemoattractant receptor structure–function relationships. Genetic manipulations of chemoattractant receptors will allow the answering of questions not otherwise approachable by studying phagocytic leukocytes themselves (i.e. structure–function relationships by deleting, adding or replacing components).

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