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# The formyl peptide chemoattractant receptor is encoded by a 2 kilobase messenger RNA

## Expression in *Xenopus* oocytes

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Activation of the formyl peptide chemoattractant receptor (FPCR) of phagocytic cells mobilizes intracellular calcium stores and affects the plasma membrane potential. Affinity crosslinking of FPCR has demonstrated a 60–80 kDa glycoprotein, with core peptide of 32 kDa. It is not known whether functional FPCR is this single peptide or requires multiple subunits. We used *Xenopus* oocyte expression system to determine the size of mRNA required for synthesis of functional FPCR. Injection of oocytes with poly(A)$^+$ RNA from HL60 cells differentiated to the granulocyte phenotype resulted in acquisition of formyl peptide-specific responses (inward transmembrane current with a reversal potential consistent with a chloride conductance, and calcium efflux). FPCR activity expressed in oocytes had a ligand concentration dependence, ligand structure dependence and pertussis toxin sensitivity similar to those reported in phagocytic cells. When RNA was size fractionated, a single peak of FPCR activity at 2 kilobases was observed after injection of mRNA into oocytes. Our data strongly suggest that FPCR is composed of a single-sized polypeptide.

Signal transduction; Chemoattractant receptor; *Xenopus* oocytes; Messenger ribonucleic acid; (HL60 cell)

## 1. INTRODUCTION

The migration of phagocytic cells from the blood to sites of infection is an essential component of host defense. This response is mediated by a number of chemoattractants including the bacterially derived formyl peptides which activate cell surface receptors that are functionally coupled to a guanine nucleotide binding regulatory protein (G-protein) [1]. Affinity labelling of the formyl peptide chemoattractant receptor (FPCR) yields a single-sized glycoprotein with relative molecular mass of 60–80 kDa. The unglycosylated core peptide is 32 kDa [2]. It is not known whether functional FPCR consists of this single affinity labelled peptide or requires additional subunits for activity.

The *Xenopus* oocyte protein expression system can be used to study heterologous G-protein coupled receptors [3,4]. We show that a phagocytic cell chemoattractant receptor, the FPCR, can be expressed in a functionally active form in *Xenopus* oocytes following injection of human myeloid cell RNA. Analysis of size fractionated RNA shows that the FPCR is encoded by a single 2 kb size class of mRNA, indicating that functional receptor likely is composed of only a single polypeptide chain.

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*Abbreviations:* cpm, counts per minute; kb, kilobases or 1000 base pairs; kDa, kilodaltons; mRNA, messenger ribonucleic acid; rpm, revolutions per minute

## 2. MATERIALS AND METHODS

### 2.1. *Reagents*

Dibutyryl cyclic AMP, *N*-formyl methionyl-leucyl-phenylalanine (fMLP), *N*-formyl norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine (f-Nle-Leu-Phe-Nle-Tyr-Lys), methionyl-leucyl-phenylalanine (MLP), *t*-butoxycarbonyl-phenylalanine-leucine-phenylalanine-leucine-phenylalanine (t-Boc-Phe-Leu-Phe-Leu-Phe) and phenylalanine-leucine-glutamic acid-glutamic acid-valine (Phe-Leu-Glu-Glu-Val) were from Sigma, St. Louis, MO. $^{45}CaCl_2$ was from New England Nuclear, Boston, MA. Pertussis toxin was a gift from Dr Munoz of the Rocky Mountain Research Laboratories of NIAID.

### 2.2. *Preparation of polyadenylated RNA*

HL60 cells were expanded and differentiated with 0.5 mM dibutyryl cyclic AMP and tested for expression of FPCR as described [2]. Differentiated but not undifferentiated HL60 cell membranes contained FPCR. Polyadenylated RNA was prepared by published methods [5].

### 2.3. *Fractionation of mRNA*

Poly(A)$^+$ RNA (500 $\mu$g) from differentiated HL60 cells was centrifuged on a 5–30% sucrose gradient formed in STE buffer (STE = 10 mM Tris, 10 mM NaCl, 1 mM EDTA, pH 7.4) in an SW41 rotor (Beckman, Fullerton, CA) at 25000 rpm for 14 h at 4°C. Fractions 1–7 and 26–34 were 400 $\mu$l each; fractions 8–25 were 250 $\mu$l each. The RNA concentration was measured on an ethidium bromide-impregnated agarose plate containing serial dilutions of a standard RNA solution. RNA was then precipitated with ethanol and dissolved

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in water at 1 ng/nl. Blot hybridization of 200 ng of RNA from each fraction was performed using $^{32}$P-labelled cDNA probes that recognize single transcripts from differentiated HL60 cells: PM1 is a 1.2 kb cDNA that recognizes a 1.2 kb transcript (Murphy and Malech, manuscript submitted). H64 (provided by Stuart Orkin) is a 2.5 kb restriction fragment of the cytochrome $b_{558}$ large subunit cDNA that recognizes a 4.5 kb transcript [6].

2.4. *Whole cell current measurements*

The maintenance of *Xenopus laevis* (Nasco, Fort Atkinson, WI) and the harvesting and microinjection of oocytes were as previously described [4]. Oocytes were injected 1 day after harvesting. Individual oocytes were voltage clamped with an Axoclamp-2 amplifier (Axon, Burlingame, CA) using a standard two-electrode voltage clamp configuration. Electrodes were filled with 3 M KCl and had resistances of 5–15 MΩ. Unless otherwise noted, oocytes were clamped at the resting membrane potential, which ranged from −55 to −75 mV. Current-voltage (I–V) relationships were obtained using 3 s voltage steps to varying potentials.

2.5. *Calcium efflux assay*

This assay was performed essentially as described [4]. Preliminary studies indicated that >90% of stimulated $^{45}Ca^{2+}$ efflux occurred within 15 min of exposure to ligand. The ligand dependent response is reported as the mean ± SE of either the net $^{45}Ca^{2+}$ efflux, calculated as the difference between the cpm detected in the 15 min stimulated extracellular fluid and the cpm detected in the final wash before addition of ligand, or as % of control, calculated as 100 × (15 min stimulated fluid cpm ÷ final wash cpm). Both treatments of the data gave similar results in all cases. Residual $^{45}Ca^{2+}$ was counted in oocyte lysates after the stimulated extracellular fluid had been removed.

## 3. RESULTS

Fig.1 shows a representative voltage clamp analysis of oocytes injected with RNA or water, and stimulated with fMLP, control peptides or stock diluent DMSO.

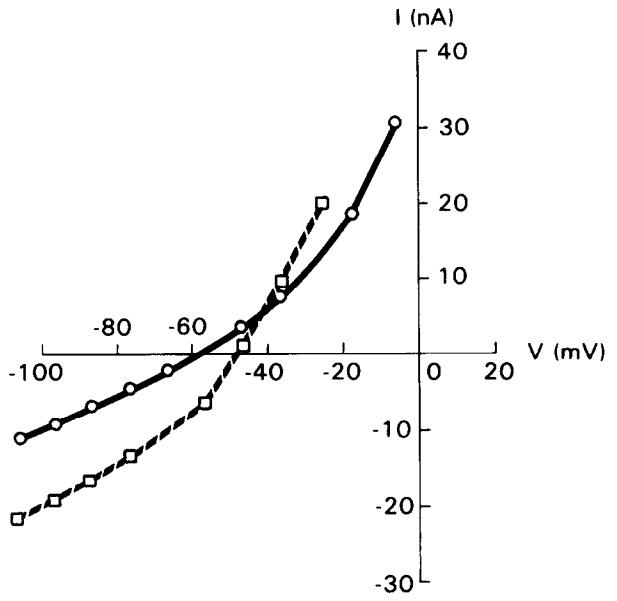


Fig.2. Current-voltage relationships obtained before (open circle, solid line) and during (open square, broken line) exposure to $10^{-6}$ M fMLP from an oocyte injected with differentiated HL60 cell RNA. Data are from the oocyte in fig.1C and are representative of 5 separate experiments.

When stimulated with fMLP, 15 of 16 oocytes injected with 50 ng of differentiated HL60 cell RNA 4 or more days prior to recording displayed an inward current response that oscillated initially and returned to baseline levels after washing the oocyte with media. The magnitude of the response for oocytes injected with 50 ng of poly(A)$^+$ RNA and stimulated with $10^{-7}$

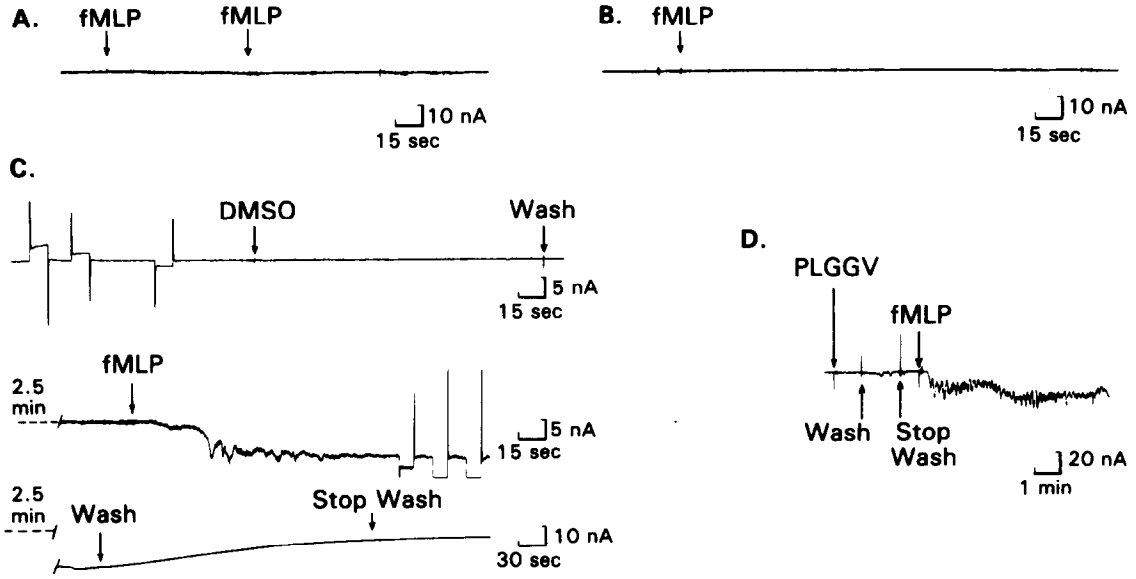


Fig.1. Acquired FPCR induced whole cell currents in *Xenopus* oocytes. Oocytes were injected with: (A) 50 nl of water (holding potential ($V_h$) = −75 mV). Arrows indicate the times of addition of $10^{-6}$ M fMLP; (B) 50 ng of poly(A)$^+$ RNA from undifferentiated HL60 cells ($V_h$ = −56 mV). $10^{-6}$ M fMLP was added at arrow; (C) 50 ng of poly(A)$^+$ RNA from differentiated HL60 cells ($V_h$ = −65 mV). DMSO 0.01% and $10^{-6}$ M fMLP were added where indicated. Current responses to voltage steps used to measure I–V relationships before and after addition of fMLP are shown; (D) 50 ng of fraction 12 (see fig.3) poly(A)$^+$ RNA from differentiated HL60 cells ($V_h$ = −60 mV). Phe-Leu-Glu-Glu-Val (PLGGV) and fMLP were added at 3 × $10^{-7}$ M. Downward deflection indicates inward current.

or $10^{-6}$ M fMLP ranged from 5 to 50 nA. This response was not present in oocytes injected with water or with 50 ng of undifferentiated HL60 cell RNA, nor was it present with DMSO or with non-formylated peptides as stimuli. *N*-formylation of peptides is a structural requirement for activation of the FPCR [7].

The I–V relationship of the fMLP-stimulated response in an oocyte injected with differentiated HL60 poly(A)$^+$ RNA is shown in fig.2. The point of intersection of the two I–V curves generated before and during stimulation is the reversal potential ($E_R$) for the fMLP-induced current response. The average $E_R$ for 5 different oocytes was $-35 \pm 4$ mV, similar to the Nernst potential for chloride and is therefore most consistent with the activation of a chloride conductance [8].

Differentiated HL60 cell poly(A)$^+$ RNA was fractionated by size on a 5–30% sucrose gradient. The average size of RNA in these fractions increased progressively in each fraction indicating that it was structurally intact (fig.3A). Moreover, the RNA could be translated by reticulocyte lysate (not shown). Fig.3B shows that peak FPCR activity measured electrophysiologically in oocytes injected with RNA was confined to a single peak at fraction 11. The peak positions in the gradient fractionated RNA of HL60 cell transcripts of known size were established by blot hybridization with 2 cDNA probes described in section 2. Based on the position of FPCR activity peak relative to the physically defined size standards, we estimate the size of the RNA encoding FPCR activity to be 2 kb.

Because the FPCR is able to mobilize intracellularly sequestered calcium in phagocytic cells, we tested RNA-injected oocytes for acquired fMLP-dependent calcium mobilization by measuring the accelerated efflux of intracellular $^{45}Ca^{2+}$. FPCR activity appears 2 days after injection of active RNA, peaks at 4 days and is still detectable at least 7 days after injection of RNA.

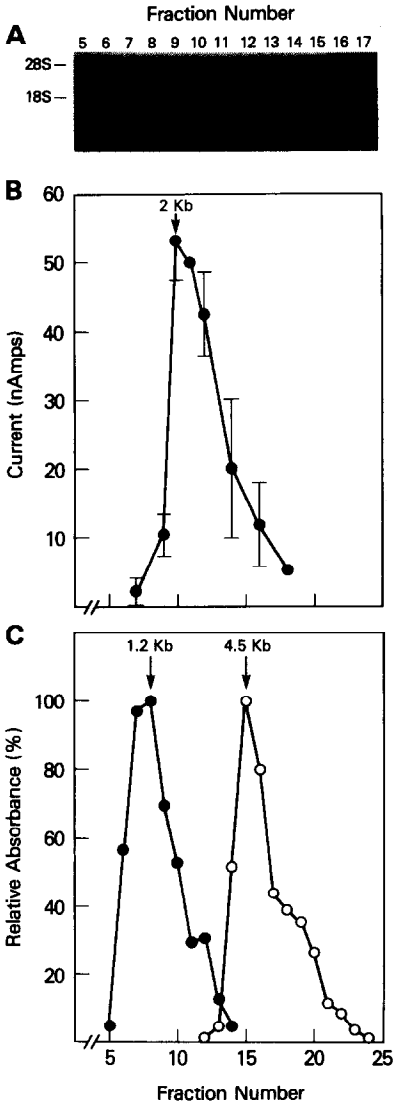


Fig.3. Size estimation of the mRNA encoding the FPCR electrogenic activity. (A) Structural integrity of sucrose gradient fractionated RNA. Poly(A)$^+$ RNA was electrophoresed on an ethidium bromide stained denaturing agarose gel. (B) FPCR activity of RNA fractions. Oocytes were injected with 50 ng of RNA from the indicated fractions and 4 days later were voltage clamped at $-60$ mV and tested with $10^{-6}$ M fMLP. Data are representative of 2 separate experiments and are expressed as the mean maximal current ± SE of 3–4 oocytes except for fractions 11 and 18 which were the mean of 2 oocytes. Fractions 5, 6, 21–31 contained no activity. (C) Sucrose gradient resolution of RNA by size. The abundance in each fraction of 2 defined transcripts from HL60 cells was determined by blot hybridization. Densitometric analysis of the autoradiographs is indicated in relative absorbance units.

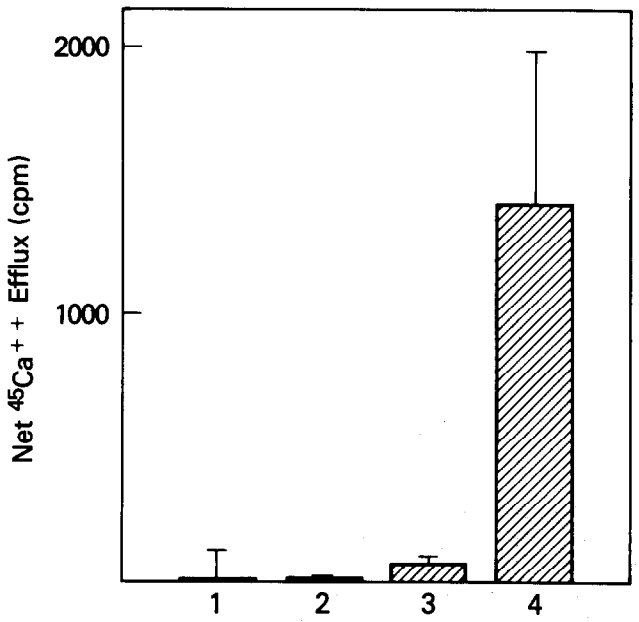


Fig.4. Enrichment of fMLP-dependent $^{45}Ca^{2+}$ efflux activity in size fractionated HL60 cell RNA. Oocytes received 50 nl of water (lane 1), 50 ng of unfractionated RNA from undifferentiated HL60 cells (lane 2) or differentiated HL60 cells (lane 3), or 50 ng of RNA from fraction 11 of sucrose gradient fractionated RNA from differentiated HL60 cells (lane 4) and were tested with $10^{-6}$ M fMLP 5 days later. Data are representative of 3 separate experiments and are from triplicate groups, 4 oocytes in each group.

The calcium response was linear for amounts of RNA injected from 3 to 50 ng (not shown). Oocytes injected with RNA from the same size fractions as those mediating the electrophysiologic responses to fMLP exhibit a greatly augmented calcium efflux response as compared to unfractionated RNA (fig.4).

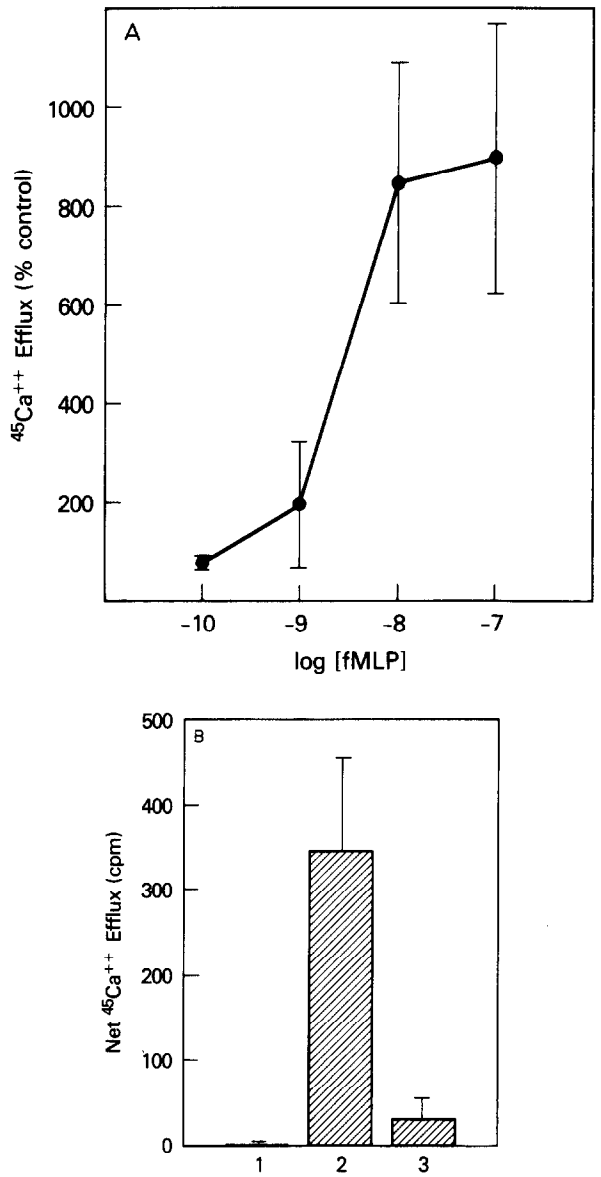


Fig.5. The FPCR calcium mobilizing activity is expressed in *Xenopus* oocytes in a physiologically faithful form. (A) Ligand concentration dependence. Oocytes were injected with 25 ng of fraction 11 RNA and were stimulated 4 days later. Data are representative of 2 separate experiments performed in triplicate, 3 oocytes per replicate. (B) Inhibition by pertussis toxin. Oocytes were injected with 50 nl of water (lane 1) or 25 ng of fraction 13 RNA (lanes 2 and 3). Five days after injection, pertussis toxin (2 $\mu$g/ml) was added to the media of half of the RNA injected oocytes (lane 3). One day later, oocytes were tested with $10^{-6}$ M fMLP. Data are representative of 4 separate experiments and are from 5 (lanes 2 and 3) or 3 (lane 1) replicates, 1 oocyte per replicate. Baseline $^{45}Ca^{2+}$ efflux between conditions differed by <2%.

We have used the calcium response to show that the physiologic properties of the heterologously expressed receptor are similar to those observed in phagocytic cells. Specifically, the ligand structure dependence is identical: non-formylated peptides such as Met-Leu-Phe, Phe-Leu-Glu-Glu-Val and t-BOC-Phe-Leu-Phe-Leu-Phe are inactive whereas fMLP and f-Nle-Leu-Phe-Nle-Tyr-Lys are active (not shown); the fMLP concentration dependence is similar to that previously reported for fMLP-stimulated calcium fluxes in human neutrophils (fig.5A) [9]; and the response is abolished by preincubation of oocytes with pertussis toxin (fig.5B). Pertussis toxin sensitivity could be partially reversed by incubating the toxin-treated oocytes in toxin-free media for an additional 24 h (data not shown).

## 4. DISCUSSION

These data demonstrate that FPCR can be expressed in a physiologically faithful form in *Xenopus* oocytes. All of the properties of active receptor are expressed after injection with RNA confined to a single sucrose gradient peak corresponding to a transcript size of 2 kb. Although it is possible that RNA encoding other signalling elements is contained in the 2 kb fraction, it has been demonstrated that the oocyte possesses native G-proteins and other more distal effector elements capable of transducing signals from heterologously expressed mammalian receptors [10]. Based on a 32 kDa unglycosylated form of the receptor that is seen by affinity crosslinking methods, a transcript containing a coding region of approximately 1 kb would be required. The data would therefore predict a combined length of 3′- and 5′-untranslated sequences of approximately 1 kb.

Our data also provide evidence that acquired FPCR transmembrane current activity in oocytes is due to activation of a chloride conductance. This raises the possibility that changes in the membrane potential of phagocytic cells stimulated with fMLP may also involve chloride conductance changes [11].

An additional implication of our study is that the oocyte expression system is a valid system for cloning cDNA encoding FPCR by the sib selection strategy, as has been done with other G-protein coupled receptors such as the serotonin-1c receptor [10]. We have recently extended this approach to examine the expression of other G-protein coupled chemoattractant receptors (manuscript in preparation). Our results suggest that this cloning strategy may also be feasible for receptors for C5a and platelet activating factor.

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