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# TRANSMEMBRANE SIGNALLING BY THE N-FORMYL PEPTIDE RECEPTOR IN STABLY TRANSFECTED FIBROBLASTS

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**SUMMARY:** We investigated the requirement for N-formyl peptide receptor-mediated transmembrane signalling in transfected mouse fibroblasts that express the receptor. Stably transfected cells displayed specific binding for N-formyl-Met-Leu-[$^3$H]Phe with a dissociation constant of 3 nM. The cells responded to ligand stimulation with mobilization of calcium from intracellular stores. Calcium mobilization was ligand dose-dependent ($EC_{50}$=3 nM fMet-Leu-Phe) and could be inhibited by pertussis toxin treatment. These results provide the first demonstration that expression of the single-chain N-formyl peptide receptor in mouse fibroblasts is sufficient for mediating ligand-induced early transmembrane signalling events, which do not appear to require other neutrophil-specific cellular components. 

Diverse neutrophil functions, including aggregation, chemotaxis, degranulation and generation of superoxide anions, can be elicited by the potent chemoattractants, N-formyl peptides. This activation process is initiated by binding of the formyl peptide ligand to specific cell surface receptor, and involves coupling of the receptor to a guanine nucleotide regulatory protein(s) (G protein), activation of phospholipase C, and generation of secondary messengers inositol 1,4,5-triphosphate ($IP_3$) and 1,2-diacylglycerol. The subsequent release of $Ca^{2+}$ from intracellular stores by $IP_3$ appears to be a primary mechanism for NFPR-mediated activation of neutrophil functions (Reviewed in Ref.1,2).

The human N-formyl peptide receptor (NFPR) has been cloned recently by screening a mammalian cell expression library for binding to iodinated formyl peptide ligand (3,4). The deduced amino acid sequence predicted a polypeptide of 350 residues with seven putative transmembrane helices, a structure conserved in G protein coupled receptors of the rhodopsin family (5). Although many of these receptors have been functionally expressed in transfected

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**Abbreviations:** NFPR, N-formyl peptide receptor; G proteins, guanine nucleotide regulatory proteins; $IP_3$, inositol 1,4,5-triphosphate; FLPEP, N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein; tBOC, t-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe; fMLP, N-formyl-Met-Leu-Phe.

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cells and in *Xenopus* oocytes, it has not been shown that the cloned NFPR can mediate transmembrane signalling when exogenously expressed. Murphy and coworkers found that *Xenopus* oocytes expressing the cloned NFPR cDNA did not respond to ligand stimulation with calcium mobilization unless messenger RNA from HL-60 cells was co-injected into the oocytes (6). This finding led to the postulation that a complementary factor might be required for the functioning of the NFPR (6). In this report, we present evidence that the exogenously expressed human NFPR is capable of mediating transmembrane signalling in mouse fibroblasts without the requirement of any other cofactor from myeloid cells.

## MATERIALS AND METHODS

**Isolation of the cDNA for human NFPR.** Poly(A+) RNA was prepared from HL-60 cells differentiated for 36 h with 500 $\mu$M dibutyryl cAMP. Double-strand cDNA was generated as described in (7). Oligonucleotide primers were synthesized based on the published human NFPR cDNA sequence (3,4) (5'-TAGGATCCAGCCATGGAGACAAATTCCTCTCTC-3', and 5'-TAAGCTTAGAATTCTACTTTGCCTGTAACGCCAC-3'), and were used in conjunction with the cDNA to amplify the coding sequence of the NFPR cDNA by the polymerase chain reaction (8). The amplified DNA (FPR1) was analyzed by DNA sequencing (9) on both strands and was shown to match the published sequence (4).

**Exogenous expression of the NFPR cDNA.** FPR1 was subcloned into the expression vector, pSFFV-neo (10). The resulting plasmid, pFPR1-neo, contained the NFPR cDNA downstream from the spleen focus-forming virus 5' long terminal repeat. Mouse fibroblasts 2071 (ATCC CCL 1.1) were transfected with 10 $\mu$g linearized vectors by the calcium phosphate precipitation method (11). Transfectants were selected by their resistance to G418 sulfate (active concentration of 0.35 mg/ml). Approximately 50-70 individual colonies from FPR1-transfected cells were pooled, generating TX2.

**Ligand binding.** Cells were harvested using trypsin-free dissociation buffer (GIBCO BRL), washed once with PBS, and resuspended to $10^6$ cells/ml in RPMI-1640 containing 0.5% BSA (binding buffer). Equilibrium binding was carried out in 0.5 ml of the binding buffer with $5x10^5$ cells and FLPEP at the desired concentration (Fig. 1), in the absence or presence of 5 $\mu$M unlabeled N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys. Following incubation for 60 minutes on ice, the cells were analyzed on a FACSCAN flow cytometer (Becton Dickinson) for fluorescent intensity. Total binding sites per cell were estimated by comparing the maximum bound fluorescent ligand determined by Scatchard analysis to standardized beads (Flow Cytometry Standards Corp.) containing 1.3-, 2.9-, 6.4-, 18- and $43x10^4$ fluorescein molecules per bead.

**Measurement of $Ca^{2+}$ mobilization.** Cells were harvested as described for ligand binding, washed once with PBS and resuspended to $5x10^6$/ml in RPMI-1640 medium containing 10% fetal bovine serum. The cells were incubated with 5 $\mu$M indo-1 AM (Molecular Probes) at 37°C for 25 minutes. Following a brief wash with the same medium, cells were resuspended to $1x10^6$ /ml in RPMI-1640 (lacking phenol red) containing 10% fetal bovine serum and maintained at room temperature until use. Continuous fluorescent measurements of calcium-bound and free indo-1 were made using an SLM 8000 photon counting spectrofluorometer (SLM-Aminco) detecting at 400 nm and 490 nm, respectively, with an excitation wavelength of 340 nm. Flow cytometric measurements were made using a FACS Analyzer (Becton Dickinson) with cells prepared as described above. Changes of calcium level detected by indo-1 were expressed as the ratio of emission at 400 nm to 490 nm. For treatment with pertussis or cholera toxins (List Biological Labs), cells were incubated with 100 ng/ml of either pertussis toxin (18 h) or cholera toxin (4 h) before harvesting for analysis.

## RESULTS AND DISCUSSION

**Ligand binding properties of the stably transfected fibroblasts.** The human NFPR was stably expressed in mouse fibroblasts. The average number of binding sites for formyl peptides ranged from 70,000 to 80,000 per cell (TX2), as determined by fluorescent intensity comparison of cell-associated N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein (FLPEP) to fluorescein-labeled standard beads using flow cytometry. This number is comparable to the average number of binding sites in neutrophils. TX2 binding of FLPEP was saturable (Fig. 1A) and was blocked by treatment of cells with the specific inhibitor, t-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe (tBOC), prior to ligand addition (not shown). Untransfected cells (Fig. 1A) or cells transfected with vector only (not shown) exhibited no significant binding of FLPEP. Scatchard analysis revealed a single class of binding site with a dissociation constant ($K_d$) of 3 nM for FLPEP (Fig. 1B). Binding analysis with N-formyl-Met-Leu-[$^3$H]Phe confirmed the $K_d$ and receptor number per cell as obtained by flow cytometry (not shown).

**Ligand-induced $Ca^{2+}$ mobilization in transfected fibroblasts.** To examine whether the exogenously expressed NFPR is capable of mediating transmembrane signalling in response to ligand binding, we used the highly sensitive $Ca^{2+}$-dependent fluorophore indo-1 to measure the intracellular $Ca^{2+}$ level in transfected cells. Cells loaded with indo-1 were monitored for fluorescence as a function of time under various stimulating conditions (Fig. 2). NFPR-transfected cells responded to fMet-Leu-Phe (fMLP) with a change in the fluorescent ratio (Fig. 2A, Tracing A). The response was observed within 2-3 seconds after ligand addition and $Ca^{2+}$ concentrations returned to near resting levels within 2 minutes. Untransfected fibroblasts (Fig. 2A, Tracing B) displayed no response to fMLP, but did respond to ATP through endogenous purinergic receptors (12). Addition of the NFPR antagonist, tBOC, did not affect ATP-induced

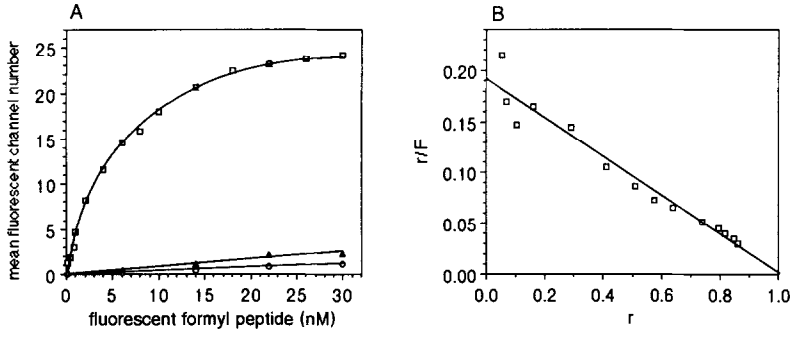


**Figure 1. Ligand binding by NFPR in transfected fibroblasts.** Binding of fluorescent ligand (FLPEP) was determined by flow cytometry. A. Ligand binding (mean fluorescent channel number) measured as a function of ligand concentration for untransfeced cells (o) and for TX2 in the absence (□)or presence (△) of 5 $\mu$M unlabeled N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys. B. Scatchard analysis of specific FLPEP binding to TX2, determined as the difference between binding of the transfected and untransfected cells. r=fraction bound; r/F=fraction bound/free.

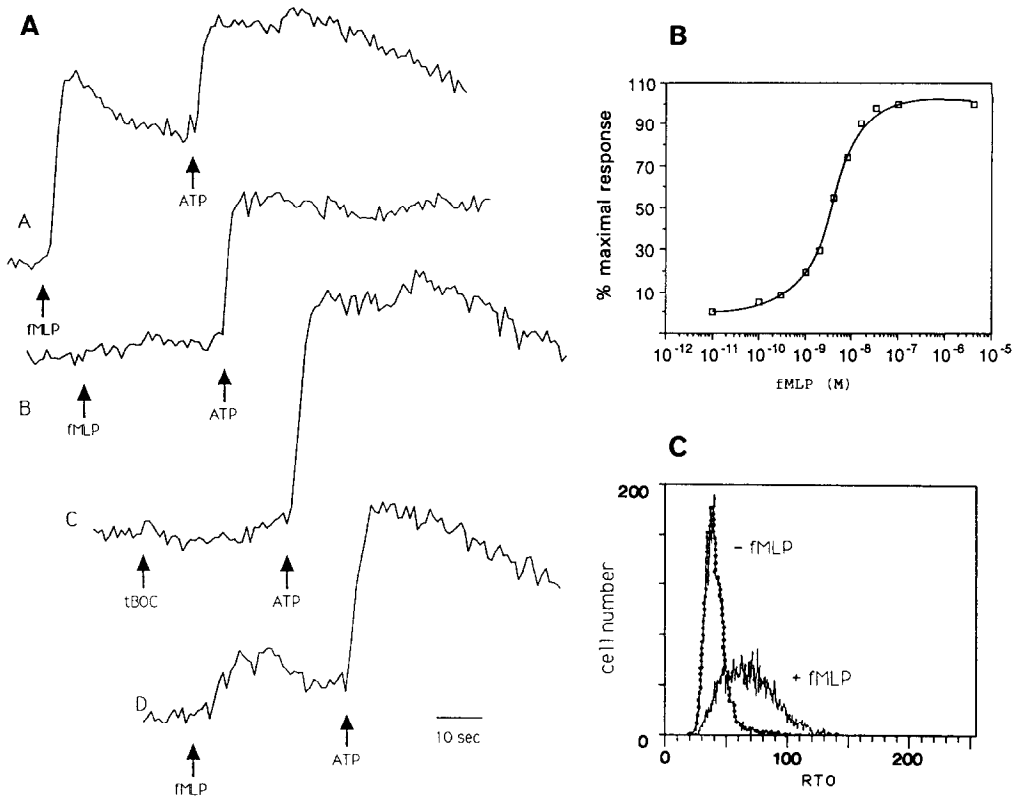


**Figure 2. Ligand-induced $Ca^{2+}$ mobilization in transfected fibroblasts.** A. Indo-1 fluorescence ratio of 400 nm/490 nm was displayed as a function of time. Arrows mark the time of addition of the indicated ligand (fMLP, 100 nM; ATP, 1 $\mu$M; and tBOC, 8 $\mu$M). The cells used are as follows: Tracing A and C, NFPR transfected cells (TX2); Tracing B, untransfected fibroblast cells; and Tracing D, TX2 preincubated for 10 minutes in the presence of 8 $\mu$M tBOC. B. Dose-dependent $Ca^{2+}$ mobilization. Peak $Ca^{2+}$ mobilization was determined as a function of the concentration of fMLP for transfected cells TX2. Values are plotted as a percentage of the maximal response. C. Flow cytometric analysis of fMLP-dependent $Ca^{2+}$ mobilization. Frequency is plotted as a function of fluorescent ratio (405/485 nm, RTO) for TX2 transfected cells with or without addition of fMLP (100 nM). The histogram is obtained from data acquired continuously from 10 to 30 seconds following the addition of ligand.

$Ca^{2+}$ mobilization (Fig. 2A, Tracing C) but significantly reduced the subsequent response to fMLP stimulation (Figure 2A, Tracing D). There was no significant change in the pattern of $Ca^{2+}$ response with addition of EGTA (10 mM) to the buffer prior to stimulation, or with the use of $Ca^{2+}$-free buffer (not shown), indicating that $Ca^{2+}$ was released from intracellular stores.

The amplitude of the $Ca^{2+}$ response is dependent on the concentration of fMLP used to stimulate the cells (Fig. 2B). Half maximal $Ca^{2+}$ mobilization was obtained at a concentration of 3 nM fMLP, representing approximately 50% receptor occupancy. With saturating amounts of fMLP (100 nM), the entire cell population responded to fMLP (Fig. 2C); whereas under the same conditions, untransfected cells demonstrated no change in fluorescence (not shown). This

result, along with the result from flow cytometric analysis of ligand binding (Fig. 1), indicates that the transfected cells responded uniformly to fMLP stimulation.

**Coupling of the NFPR to an endogenous pertussis toxin-sensitive G protein in transfected fibroblasts.** In neutrophils and differentiated HL-60 cells, NFPR mediates signal transduction by coupling to a G protein $\alpha$-subunit that is a substrate for ADP-ribosylation by pertussis toxin (13). Both $G_{i2}$ and $G_{i3}$ have been implicated to be involved in this signalling process (14), resulting in stimulation of phospholipase C activity. To determine whether the same coupling mechanism is involved in the transfected fibroblasts, we examined the sensitivity of the ligand-stimulated $Ca^{2+}$ mobilization to treatment with pertussis toxin. Such treatment completely abolished the $Ca^{2+}$ mobilization in response to fMLP (Fig. 3). The specificity of the inhibition by pertussis toxin was indicated by the lack of an effect on $Ca^{2+}$ mobilization generated by ATP through the endogenous fibroblast purinergic receptor, which mediates its response through both pertussis toxin sensitive and insensitive G proteins (13,15). Treatment of the transfected fibroblasts with cholera toxin (100 ng/ml for 4 hours) had no effect on fMLP-induced $Ca^{2+}$ mobilization (not shown).

These results demonstrated for the first time that exogenous expression of the human NFPR in mouse fibroblasts reconstitutes ligand-specific transmembrane signalling, as reflected by the release of $Ca^{2+}$ from intracellular stores. This response appears to be mediated by the functional coupling of the expressed NFPR to an endogenous pertussis toxin-sensitive G protein, probably $G_{i2}$, since mouse fibroblasts contain a $G_{i2}$ protein that interacts with the mannose 6-

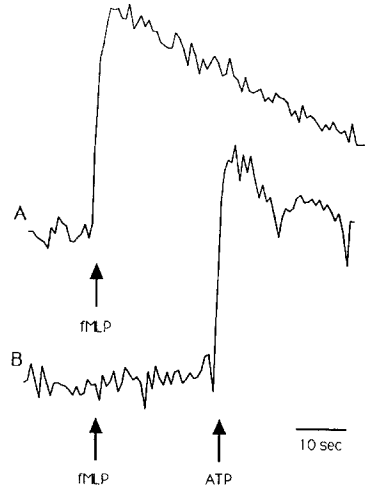


**Figure 3. Effect of pertussis toxin treatment on $Ca^{2+}$ mobilization in transfected fibroblasts.** Near confluent fibroblast cells were grown in the absence (Tracing A) or presence (Tracing B) of pertussis toxin (100 ng/ml) for 18 h prior to harvesting. Arrows indicate the addition of either fMLP (100 nM) or ATP (1 $\mu$M).

phosphate/IGF-II receptor (16). Ligand-induced $Ca^{2+}$ mobilization in transfected fibroblasts is rapid and dose-dependent, with $EC_{50}$ of 3 nM fMLP. This is similar to data obtained from experiments using neutrophils. Collectively, results from our experiments indicate that functional coupling of G protein to the recombinant NFPR can occur between different species, and has no requirement for the participation of other neutrophil-specific components. In contrast, Murphy and coworkers reported that functional expression of the human NFPR in *Xenopus* oocytes required a complementary factor from HL-60 cells (6). Although the nature of this complementary factor is unclear at present, our data suggest that it exists in non-myelocytic cells and therefore is not a neutrophil-specific component. We have recently established a 293 cell line that express the human NFPR and found it, too, responded to fMLP stimulation with $Ca^{2+}$ mobilization. These cell lines should prove to be useful tools for further studies on the mechanisms of NFPR-G protein interaction.

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