Short communication

Characterization of chemotactic ability of peptides containing N-formyl-methionyl residues in *Tetrahymena* fMLP as a targeting ligand

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Abstract

The chemotactic effects of six formylated, putatively bacterial peptides (fMLP, fMLPP, fMMM, fMP, fMV, and fMS) were studied. From the set of six peptides, only fMLP (one of the most effective chemoattractant peptides in mammals) elicited a significant positive chemotactic response in the eukaryotic ciliate *Tetrahymena pyriformis*, while the other formylated ligands, e.g. fMMM (which is also effective in mammals), had neutral or antagonistic effects in *Tetrahymena*. A study of their amino acid sequences points to an, as yet obscure, interaction between C-terminal f-Met and N-terminal aromatic Phe. Some optimal physicochemical characteristics (e.g. solvent exposed area, solubility) of the molecule may be responsible for this special feature of f-MLP at such a low level of phylogeny. This means that the unicellular *Tetrahymena* is able to select between related molecules, giving high priority to the molecule that is the most chemoattractive in mammals. The results call attention to the possible presence of f-Met receptors at a unicellular level and to the evolutionary conservation of chemotaxis-activating processes.

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Keywords: Chemotaxis; Formylated peptides; Selection; Targeting; *Tetrahymena*; Phylogeny

1. Introduction

Chemotaxis is one of the most fundamental physiological responses that developed at prokaryotic and early eukaryotic levels of phylogeny. The two distinct levels possess diverse signalling mechanisms. Interaction of a well described receptor–intracellular signalling system (e.g. Trs, Trg receptors and the ChA–ChB–ChY–ChZ network) and several relatively simple ligands (amino acids, dipeptides, saccharides) are present in bacteria (Parkinson, 1993). In contrast, different membrane associated receptor complexes and their still poorly understood downstream signalling networks have been described in eukaryotic cells, e.g. chemokine receptors (Rossi and Zlotnik, 2000), G-protein linked signalling (Ridley, 2001). The receptor-mediated responsiveness of both unicellular levels is essential, as it allows detection of harmful and beneficial ligands (e.g. toxins and nutrients) alike.

Besides these independent prokaryotic or eukaryotic systems, some bacterial products have strong chemoattractant ability in multicellular organisms; several pathogenic bacteria (such as *Escherichia coli* or *B. subtilis*) produce such substances. Pioneer work by the Freers group proved that several N-formyl methionyl peptides synthesized by bacteria possess chemoattractant abilities (Schiffmann et al., 1975). Modification of the C-terminal part of these peptides, especially with Met or Phe residues, could result in characteristic changes in chemotactic potency or release of lysosomal enzymes in neutrophil granulocytes (Freer et al., 1980). Among these products, short peptides with the N-formyl methionyl residue possess particularly strong chemo-
N-For–Met–Leu–Phe (hereafter fMLP) is one of the strongest reference chemotaxant substances used in chemotaxis experiments with neutrophils (Showell et al., 1976). However, receptors of fMLP are also expressed in other white blood cells and another large group of mammalian cells, i.e. cells of the lamina propria of interstitial mucosa (Anton et al., 1998).

Investigations of this chemotaxant molecule show that chemotactic responsiveness to fMLP has a strong correlation with phosphoinositide 3-kinase signalling (Pellegatta et al., 2001), intracellular calcium release (Verploegen et al., 2002), adhesion (Willeke et al., 2001), and F-actin co-localization to the leading edge of polarized neutrophils (Howard and Oresajo, 1985). Further characterization of G protein-coupled fMLP receptors have revealed that, in addition to the above-mentioned components, all signal transduction networks (phospholipase A2, C, D; Ser/Thr or Tyr kinases; ion channels) and several chemotaxis related functions (degranulation, oxidant production, release of pro-inflammatory mediators) are activated (Murphy, 1996).

Results of other experiments have shown that there are significant homologies of chemotactic signalling in relatively simple eukaryotic members of phylogeny. The unicellular Tetrahymena pyriformis recognizes similar ligands, e.g. IL-8 (Kohidai and Csaba, 1998), endothelin-1 (Kohidai and Csaba, 1995) or insulin (Csaba et al., 1994), and responds in the same concentration range and direction as vertebrate model cells. Former results have shown that Tetrahymena membrane has a high affinity ($K_D=4 \times 10^{-9}–10^{-8} \text{ M}$) for fMLP (Leick, 1992), and other derivatives possessing the formyl group (For–Nle–Leu–Phe and BOC–f Nle–Leu–Phe) also have characteristic and competitive chemotaxant effects in this protozoa (Kohidai et al., 1994). Whilst the relationship of the molecular composition of these bacterial peptides with their chemotactic potency has been investigated in migratory cells of vertebrates (Schiffmann et al., 1975), data on eukaryotic protozoa is still lacking. The wide range of homologies and sensitivity of Tetrahymena to slight structural modifications in the relatively short and long chemotaxant peptide type ligands, e.g. proline-containing dipeptide (Kohidai et al., 1997) or insulin derivatives (Csaba et al., 1994), is another reason for using this unicellular organism in our experiments.

In the present study, our objectives were whether:

1. different bacterial formylated peptide derivatives are also chemotactic to eukaryotic ciliates (such as Tetrahymena)
2. there is any correlation between the composition of formylated peptides and their chemotactic potency
3. the chemotactic behavior and selectivity of Tetrahymena can be compared to that of mammalian granulocytes

For this purpose, six formyl-methionyl derivatives were assayed in T. pyriformis GL.

2. Materials and methods

2.1. Cell culture

Populations of T. pyriformis GL taxon in logarithmic phase of growth were cultured in 0.1% yeast extract containing 1% Bacto Tryptone (Difco, Michigan, USA) at 28 °C. Prior to the experiments, cells were transferred to Losina Losinsky (LL) medium (Losina-Losinsky, 1931) containing inorganic salts (see below), for 3 h. Cell density of LL cultures was $10^6$ cell/ml.

2.2. Chemicals and buffers

Six N-formyl methionyl peptide derivatives were tested: For–Met–Leu–Phe (fMLP), For–Met–Leu–Phe–Phe (fMLPP), For–Met–Met–Met (fMMM), For–Met–Phe (fMP), For–Met–Val (fMV) and For–Met–Ser (fMS) were obtained from Sigma, St Louis, USA. Fmoc–Ser(tBu)–Wang resin, fluorenymethylmethionine (Fmoc-Met), dimethyl formamide (DMF), piperidine, methanol (MeOH), dimethyl formiate, triethyl silane (TES), trifluoroacetic acid (TFA) and ethyl-dimethylaminopropyl carbodiimide (EDCI) were products of Fluka Sigma–Aldrich Ltd, Budapest, Hungary. Effects of the amino acid residues of the formylated oligopeptides (For–Met, Met, Leu, Phe, Ser and Val) mentioned above were also assayed (all these amino acids were also obtained from Sigma, St Louis, USA). Composition of LL solution was 1% NaCl; 0.1% MgCl₂; 0.1% CaCl₂; 0.1% KCl and 0.2% NaHCO₃.

2.3. Synthesis of For–Met–Ser

Fmoc–Ser(tBu)–Wang resin (0.1 mmol, 200 mg) was swollen in DMF ($3 \times 3$ ml, $3 \times 1$ min), incubated with piperidine/DMF (1:1 v/v, $3 \times 3$ ml, 2+10+5 min), washed with DMF, MeOH and DMF ($3 \times 3$ ml and 1 min each). Reagents and solvents were removed by suction in every case. Fmoc–Met (0.3 mmol, 112 mg) was dissolved in DMF (2 ml) then DIC and HOBt (0.3 mmol each, 47 µl and 46 µl, respectively) were added. The solution was allowed to stand for 5 min and added to the resin. The slurry was shaken at RT for 2 h. The resin was washed with $4 \times 3$ ml DMF then deprotected with piperidine/DMF (see above). The peptide on the resin was then formylated with $p$-nitrophenyl formiate (Martinez and
Laur, 1982). The reagent (0.4 mmol, 80 mg) was dissolved in 0.6 ml DMF, for 3 h at RT. The resin was washed with DMF then MeOH, and dried over P2O5. The peptide was cleaved from the resin using a mixture of TFA, TES and water (90:5:5, v/v/v). The mixture (8 ml) and the dry resin were mixed in the cold (0 °C) and stirred at RT for 3 h, then filtered. The filtrate was evaporated in vacuo, the remainder was dissolved in water, freeze-dried and purified by HPLC, using a Phenomenex Jupiter C18 (100 A, 5 µm) column (250 × 10 mm). The structure was confirmed by ESI-MS with a Perkin–Elmer Sciex API2000 tandem mass spectrometer. M (theoretical)=264.3; M+ (actual)= 265.0.

2.4. Chemotaxis assay

The chemotactic ability of *Tetrahymena* cells was evaluated using a modified two-chamber capillary chemotaxis assay (Kohidai et al., 1995). Tips of an eight-channel micropipette filled with the test substances served as the inner chamber of the system. The outer chamber consisted of a microtitration plate filled with the model cells. The incubation time was 20 min. This relatively short time facilitated the measuring of pure gradient, directed chemotactic responses and prevented contamination of the samples from randomly running chemokinesis. The incubation medium was 0.9% NaCl solution and the neutral character of dipeptide fMP also shows a good overlap of chemotactic responsive structures (Fig. 1a). Elongation of the N terminus, only fMLP (the “classic” chemoattractant) could elicit dose-dependent chemotactic responses with a bell-shaped curve in *Tetrahymena*. Its most effective chemoattractant concentration range was 10⁻¹² to 10⁻⁶ M, although a slight chemoattractant activity was detected up to 10⁻⁶ M (Fig. 1a). Elongation of the C-terminus with an additional Phe residue (fMLPP) resulted in a diminished, neutral or slightly depressed chemoattractant response over a wide (10⁻¹¹–10⁻⁶ M) concentration range, while the lowest concentration tested (10⁻¹² M) was chemorepellent (Fig. 1b). In the case of the tripeptide composed of only methionines (fMMM), neutral or slightly depressed chemotactic activity was detected (Fig. 1c). The Leu-deletion variant dipeptide (fMP) showed that not only elongation, but also omission of fMLP, can result in the loss of chemoattractant ability, as fMP had a neutral character at all the tested concentrations (Fig. 1d).

The other two dipeptides (fMV and fMS) were assayed to test whether a combination of fMet with different amino acid residues on the C terminus could modify the chemotactic ability of the peptide. The chemoattractant character of fMV at 10⁻⁶ M (Fig. 1e) and the significant chemorepellent feature of fMS over a wide range (10⁻¹²–10⁻⁸ M) (Fig. 1f) suggest that a low solvent exposed area (SEA) (Rose et al., 1985) in the C terminal amino acid (Val=23.5) is preferred over higher values (Ser=44.2 or Phe=28.7). We could conclude that both strong (Phe=2.87) and weak (Ser=0.07) hydrophobicity at the C terminus are disadvantageous with regard to chemoattractant ability, while a moderately hydrophobic (Val=1.87) C terminus results in a chemoattractant formylated dipeptide ligand (Jones, 1975).

The concentration range studies showed that slight modifications to the short formylated (putatively bacterial) peptides can alter their chemotactic character, however, a few peptides possess some chemotactic ability (e.g. fMV as a chemoattractant and fMS as a chemorepellent), hence the identity of fMLP seems to be the result of a sensible structure-function relationship.

The chemoattractant or repellent characteristics of the peptides described above may be explained by some of their physicochemical properties. As previous studies have also described, chemotactic characteristics of short peptides show good correlation with SEA values or solubility of terminal residues of short peptides (Illyés et al., 2002). Our current results indicate that optimal values of mass, SEA or ratio of SEA:solubility are advantageous with respect to the chemoattractant ability of the ligand. Table 1 shows that fMLP possesses intermediate values; the higher or lower values are not favored in chemotactic signalling.

Comparison of our results on fMLP in *Tetrahymena* with Freers data obtained in neutrophils (Showell et al., 1976) shows a good overlap of chemotactic responsiveness in protozoa and neutrophils (both 10⁻¹⁰–10⁻⁸ M). The neutral character of dipeptide fMP also shows a good correlation at both levels of phylony. In the case of peptide fMMM, significant differences were observed, as this compound exhibits a slight chemorepellent effect over a wide concentration range in *Tetrahymena*, in contrast to its strong chemoattractant effect in vertebrate neutrophils. These data suggest that phylogeny of
signalling in this family of chemoattractants includes continuity and divergence. Peptide fMLP is utilized in unicellular and multicellular organisms by similar effector mechanisms, while signalling of fMMM probably uses different effector mechanisms on the two levels. However, it must also be considered that fMLP is a real bacterial peptide (Broom et al., 1992; Fontan et al., 1992; Le et al., 2002; Mooney et al., 1991), while fMMM (and the others used) are synthetic peptides that may or may not be produced by bacteria. Therefore, *Tetrahymena* and neutrophils appear to react to the “natural” peptide and reject the synthetic ones.

### Table 1

<table>
<thead>
<tr>
<th>Mass</th>
<th>SEA</th>
<th>SEA/Solubility of C-terminal residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>f-MV</td>
<td>230.33</td>
<td>54.0 6.10</td>
</tr>
<tr>
<td>f-MP</td>
<td>278.37</td>
<td>59.2 19.96</td>
</tr>
<tr>
<td>f-MS</td>
<td>218.27</td>
<td>74.7 14.87</td>
</tr>
<tr>
<td>f-MLP</td>
<td>391.53</td>
<td>88.2 29.74</td>
</tr>
<tr>
<td>f-MMM</td>
<td>393.59</td>
<td>91.5 27.06</td>
</tr>
<tr>
<td>f-MLPP</td>
<td>538.70</td>
<td>116.9 39.42</td>
</tr>
</tbody>
</table>

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### 3.2. Relationship between the chemotactic effect of amino acids and peptides

In the case of bacterial peptides, the expression of N terminal For–Met moiety is characteristic and functionally significant; in several cases (e.g. B. licheniformis), only the N-formylated precursor holoproteins can induce neutrophils, in contrast to the mature, N-formylated signal-lacking proteins (Bennett et al., 1980). Our first comparative study was to analyze the chemotactic ability of this structurally modified amino acid in parallel with methionine. Although both compounds were chemoattractant (Fig. 2), the presence of the formyl group itself did not improve chemotactic potential. Moreover, native methionine was more potent at 10^{-10} M in *Tetrahymena*.

Data in the literature document relationships between the chemotactic character of short peptides and their amino acid constituents (Almagor et al., 1981). Therefore, the chemotactic character of the remaining four amino acids of the tested peptides was also analyzed (Fig. 2). Our data show that Leu, Phe and Val have a wide-ranging (10^{-12}-10^{-6} M) chemorepellent effect. Only Ser displays a biphasic concentration dependence, being chemoattractant at low (10^{-12}-10^{-10} M) and chemorepellent at higher (10^{-6} M) concentrations.
These results confirm other data in the literature that, even in short peptides (di- or tripeptides), the chemotactic character of the molecule cannot be derived from the features of the constituent amino acids. In spite of the fact that fMet is moderately chemoattractant in character and amino acids Leu, Phe and Val are strong repellents, peptides derived from these units form a diverse set of ligands. In peptides, their physicochemical characteristics (SEA, etc.) seem to be decisive.

In conclusion, our investigations on the chemotactic effects of N formyl-methionine-containing peptides suggest that fine, intramolecular physicochemical adjustments are required for the chemoattractant effect of the well-known reference peptide fMLP. Our results show that signalling of fMLP is a well-conserved mechanism in phylogeny, while other formylated ligands have neutral or antagonistic effects in low and higher levels of eukaryotes. The results clearly show that the chemotactic reaction and sensitivity of human granulocytes to chemoattractants can be traced back to the early stages of phylogeny, however, the repertoire of response-ability has been modified over time. The experiments also demonstrate that Tetrahymena can very keenly select between related peptides, which results in a preference for fMet receptors even at this low level of phylogeny.

Acknowledgements

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References


