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Chemotactic activity of oligopeptides containing an EWS motif on *Tetrahymena pyriformis*: the effect of amidation of the C-terminal residue

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Chemotactic properties of 3–7-mer peptides containing an EWS motive and their peptide amides synthesized and characterized by us were investigated in *Tetrahymena pyriformis GL* model. Analysis of the peptide acids shows that SEWS possesses exceptionally strong (660% ± 21; 430% ± 18) chemoattractant ability at 10^{-12} and 10^{-11} M respectively. The shorter peptide (EWS) possesses chemorepellent activity, while longer peptides display neutral (WSEWS) or moderate chemoattractant (EWSEWS and GEWSEWS) chemotactic ability. Amidation of the C-terminus can significantly modify the character of peptides: it points to the conclusion that a free α-COOH group at this position is required for the high efficiency of SEWS, while in the shorter (EWS) and longer peptides (WSEWS and EWSEWS) amidation can result in chemoattractant ligands. Evaluation of the structure–function relationship of these compounds establishes the significance of Glu (E) with its high surface-exposed area and negatively-charged side chain. The high discriminative ability and good chemotactic responsiveness of *Tetrahymena* support the theory that a chemotactic signalling mechanism working in higher levels of phylogeny is a well conserved and inducible one even in protozoa.

KEY WORDS — chemotaxis; oligopeptides; peptide amides; SEWS; signalling; *Tetrahymena*

INTRODUCTION

Chemotaxis is one of the most ancestral and essential physiological responses of cells. Its phylogenetic background is based on the early phases of ligand–receptor interactions when simple substances consumed as nourishment and special ligands possessing additional effects on metabolic processes, were selected in a parallel process of ligand and its receptor.[1] Data in the literature indicate that chemotactic receptors are extremely sensitive as they can distinguish even D or L isomers of amino acids[2] or oligopeptides with or without a formyl group at their N-terminus.[3] Chemical modification (acetylation, methylation or periodate treatment) of chemotactic ligands derived from bacteria also results in significant loss (25–85%) of chemotactic potency.[4] Other physicochemical characteristics e.g. residue size or lipophilicity of dipeptides at the N-terminus can also influence chemoattractant features of the ligand.[5] However, our knowledge concerning the structure–function relationship of such ligands is still incomplete. The objective of the present work was to synthesize oligopeptides with 3–7 amino acid residues containing an EWS motif and to evaluate whether (i) N-terminal elongation of the EWS tripeptide can change the chemoattractant potency of the ligand; and (ii) amidation of the carboxy-terminus of these ligands can result in concordant loss of chemotactic activity comparable with other kinds of chemical modifications described in the literature.[4]

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The significance of the EWS motive selected in biology and especially in immune responses, is underlined by its presence within one of the extracellular WSXSW domains of a cytokine receptor superfamily, including IL-2, IL-4 and IL-6 receptors.[9] The classical chemotactic effect and induction of haptotaxis are conceivable. Soluble receptors of IL-6 can induce migration of cells in the fluid phase, while segments of the membrane-bound receptor may induce the cell surface-linked form of migration, haptotaxis.[6]

In order to investigate the chemotactic properties of this new family of peptides, the unicellular eukaryotic ciliate *Tetrahymena pyriformis*, a frequently used protozoon in molecular and cell biology,[7] was used as a model. The membrane receptors e.g. insulin[8] or opiate receptors.[4] second messenger systems e.g. cAMP,[9] IP_3[10] or Ca^{2+}-calmodulin[11] and intracellular metabolic pathways,[12] show close homologies with higher ranked animals. In respect to chemotactic signals. *Tetrahymena* has also proved to be highly sensitive to chemical structure and concentration-dependent effectiveness of different groups of ligands e.g. inorganic salts,[13] amino acids,[14] peptide hormones, cytokines and lectins.[15,16] Using this model, slight structural differences in ligands have been shown to be discriminated as chemoattractant or chemorepellent agents.[17] In addition to the evaluation of the chemical modification of chemotactic oligopeptide ligands, the well-described protozoan model provided us a suitable experimental model from which to gain new data concerning phylogenetic backgrounds of signalling in chemotaxis.

MATERIALS AND METHODS

Materials

Wang resin ($0.96\,mmol\,g^{-1}$) was obtained from Bachem, Bubendorf, Switzerland (Cat. No. D 1250: lot 504143): AM-RAM resin ($0.81\,mmol\,g^{-1}$) was a product of Rapp Polymere, Tübingen, Germany, Fmocamino acids were purchased from Fluka, Buchs, Switzerland, Reagents *N,N′*-diisopropylcarbodiimide (DIC), dimethylaminopyridine (DMAP), I-hydroxybenzotriazole (HOBt), piperidine, trifluoroacetic acid (TFA)) and solvents, dimethylformamide (DMF) and methanol were Fluka products and of analytical grade.

Synthesis of peptides

Coupling of Fmoc-(^{t}Bu)Ser to the Wang resin was performed with DIC using DMAP as the acylating catalyst.[18] Wang resin (1 g, 0.96 mmol) was swollen in 10 ml DMF then Fmoc-(^{t}Bu)Ser (1.15 g, 3 mmol), DIC (0.47 ml, 3 mmol) and DMAP (37 mg, 0.3 mmol) were added. The mixture was stirred for 3 h at room temperature (RT) and washed with DMF, EtOH and DMF. The whole coupling procedure was repeated. When using AM-RAM resin for preparation of peptide amides, after swelling, the resin was treated with a mixture of piperidine and DMF (1:1, v/v: 2 and 9 min) then the coupling procedure was identical with that described above. There was no need, however, for the repetition of coupling.

For the synthesis of the oligopeptide chain 50–100 mg of Fmoc-(^{t}Bu)Ser-Wang resin (35–70 μmol) or 50–100 mg of Fmoc-(^{t}Bu)Ser- AM-RAM resin (30–60 μmol) was treated with 3–5 ml of reagent solution or washing solvent. The resin was swollen with DMF (3×2 min). The Fmoc group was removed by reacting the resin three times with a mixture of piperidine and DMF (1:1, v/v: 1, 9 and 1 min) followed by washing with DMF (3×1 min), MeOH (2×1 min) and DMF (4×1 min). In the course of coupling of the next amino acid residue, 200% molar excess of Fmoc-amino acid and of HOBt[19] and DIC[20] were dissolved in 2.0–2.5 ml DMF. The solution was allowed to stand for 5 min, added to the resin and the slurry was shaken for 90 min. The resin was then washed with DMF (4×2 min) and MeOH (2×2 min). The conversion was monitored with a ninhydrin test.[21]

After a final deprotection step followed by washing and drying, the oligopeptide was cleaved from the resin. Peptidyl resin (50–100 mg) was stirred with 3–5 ml of a mixture of TFA (95%), ethanedithiol (2.5%) and water (2.5%) for 3 h at RT. The resin was filtered off and the filtrate containing the desired product was precipitated with ether and centrifuged in a sealed tube. The precipitate was washed three times with ether, dissolved in water or diluted in acetic acid (AcOH) and freeze dried.

Analytical reversed-phase high pressure liquid chromatography (RP-HPLC) experiments were carried out using a DeltaPak C18 (300×3.9 mm) column (300 Å, 15 μm) with a flow rate of $1.0\,ml\,min^{-1}$ at RT. The peptides were purified by using a semi-preparative DeltaPak C18 (300×15.0 mm) column (300 Å, 15 μm) at a flow rate of $4.0\,ml\,min^{-1}$ on a Knauer HPLC system (Bad Homburg, Germany). Eluants were: A, 0.1% TFA in water and B, 0.1% TFA in acetonitrile/water (80:20, v/v). A linear gradient of eluant B was used.

Fast atom bombardment mass spectroscopy (FAB-MS) experiments were performed with a Fisons VG ZAB-2SEQ hybrid tandem mass spectrometer of BEQQ configuration (Loughborough, UK) equipped with a liquid secondary ion mass spectrometer source (Cs^+ ion gun used at 30 keV) and coupled to an OPUS

2000 data system. The samples dissolved in 0.05 M NH_4HCO_3 buffer containing 0.1% TFA were mixed with glycerol before being subjected to FAB-MS analysis.

Cells and culturing

Tetrahymena pyriformis GL cells, maintained in 0.1% yeast extract containing 1% Bacto tryptone (Difco. Michigan, USA) medium at 28°C were used in the logarithmic phase of growth. The density of samples was 10^4 cell ml^{-1}.

Assay of chemotaxis

The chemotactic ability of cells was determined in a two-chamber capillary assay system of Leick and Helle[22] modified by us.[23] According to this set-up, tips of a multi-8-channel automatic pipette served as an inner chamber to minimize the standard error of sampling, while microtitration plates were used as outer chambers. The outer chamber was filled with the cells to be tested, the inner one contained the test substance with different concentrations (10^{-12}–10^{-6} M) of the peptides. (In the case of SEWS the concentration range was extended to 10^{-16}–10^{-6} M, due to the exceptionally high effectiveness at the usual lowest threshold). In control experiments, culture medium was used as attractant (negative control). For validation of the assay, two chemoattractants f-Met-Leu-Phe (f-MLF; Sigma, St. Louis, USA) and interleukin-8 (IL-8; Promega, Madison, USA) were also tested as positive controls. After 15 min incubation the samples in the inner chambers, containing the chemotactically positive responder cells, were fixed in 4% formaldehyde containing PBS, (0.05 M phosphate buffer, pH 7.2; 0.9 M NaCl). The samples were evaluated in a Neubauer haemocytometer.

Statistical evaluation of data

Each peptide was tested in 10 replicate assays, the figures demonstrate the averages of these results. The statistical analysis was carried out using ANOVA of Origin 4.0.

RESULTS

Solid phase synthesis and characterization of peptides and peptide amides

The Fmoc/^{t}butyl technique[24,25] was used for building up linear oligopeptide chains with an *in situ* active ester (HOBt/DIC) coupling strategy. The synthetic protocol is described in the experimental section. For side-chain protection of Ser and Glu, ^{t}butyl ether and ^{t}butyl ester were utilized, respectively. No blocking group was applied for the indole ring of Trp. The crude product was analysed by RP-HPLC and target peptides were isolated. As indicated by mass spectrometry (MS) and retention time (R_t) data summarized in Table 1, peptides with high purity were used for the biological evaluation.

Table 1. Chemical characterization of peptides and peptide amides with the EWS motif

Peptide	R_t*	Relative molecular mass	
	(min)	Calc.	MH^- obs.
EWS	6.1*	420.2	420.9
SEWS	10.7*	507.2	507.9
WSEWS	16.0†	693.3	694.9
EWSEWS	17.5†	822.3	823.7
GEWSEWS	20.5‡	879.3	880.5
EWS-NH_2	6.6*	419.2	419.7
SEWS-NH_2	10.0*	506.2	506.7
WSEWS-NH_2	16.0†	692.3	693.7
EWSEWS-NH_2	17.0†	821.3	822.5
GEWSEWS-NH_2	22.5†	878.3	879.5

HPLC was performed on a C18 DeltaPak 300 L, 15 µm, 300 × 3.9 mm column, with a flow rate of 1.0 ml min^{-1} at RT, detection at 220 nm. Eluants: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile/water (80:20, v/v). Relative molar mass was determined by FAB mass spectrometry.
*B 1–55% in 25 min; †B 15–45% in 30 min; ‡B 5–55% in 25 min.

Chemotaxis induced by the peptides and peptide amides

Results of dose–response studies elicited by synthetic peptides and peptide amides in chemotaxis assays are shown in Figures 1–5. Comparison of chemotactic effects was undertaken to identify peptides with chemotactic activity within the set of oligomers containing the EWS motif and to analyse the effect of N-terminal elongation. In the second part of our study we have compared the activity of peptide acid with that of peptide amide under identical experimental conditions to gain a better understanding of the influence of amidation on chemotactic activity.

The shortest peptide tested was the tripeptide EWS. Peptide EWS with a carboxyl group at its C-terminus showed a strong chemorepellent activity in a wide concentration range (10^{-10}–10^{-6} M; Figure 1a). At lower concentrations this effect was not observed. Interestingly the amidated variant of EWS expressed significantly divergent chemotactic potencies

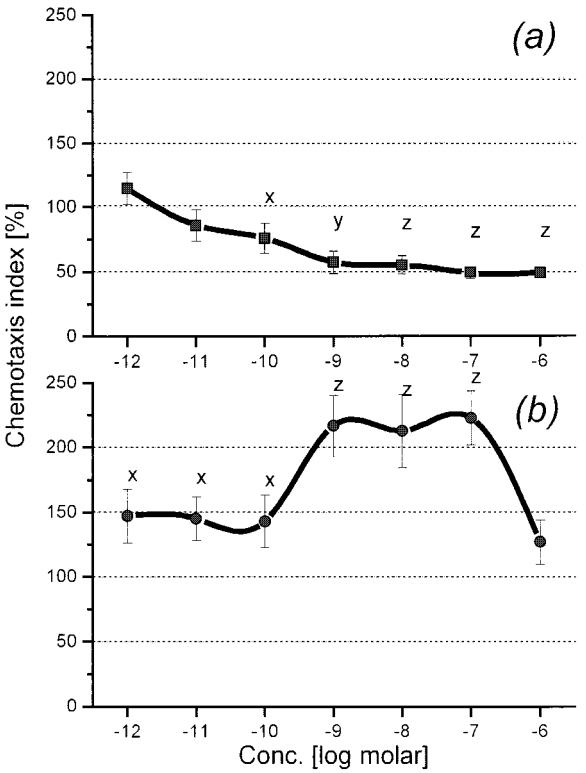

Figure 1. Dose–response correlation of chemosensory responses elicited in *Tetrahymena pyriformis* GL with EWS peptide (a) and its peptide amide (b). x, $p < 0.05$; y, $p < 0.01$; z, $p < 0.001$

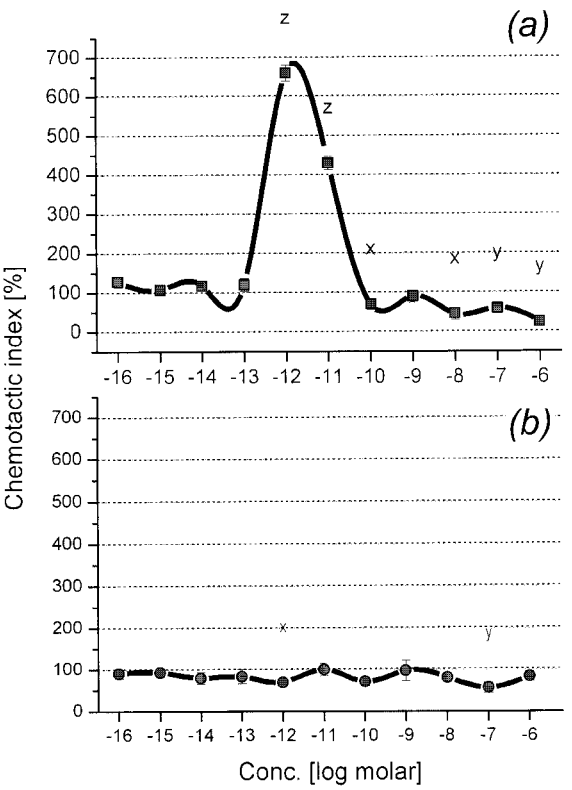

Figure 2. Dose–response correlation of chemosensory responses elicited in *Tetrahymena pyriformis GL* with peptide SEWS (a) and its amide (b). x, $p < 0.05$; y, $p < 0.01$; z, $p < 0.001$

(Figure 1b). In high concentrations (10^{-9}–10^{-7} M) the peptide had high chemoattractant effect on *Tetrahymena* cells (210–221%). The effect can be considered as markedly significant, since the peptide acid (EWS) was chemorepellent in the same concentration range. The chemoattractant feature of the amide variant of EWS was also detectable at the lower concentrations.

In the case of SEWS (Figure 2a) the native peptide with COOH at the C-terminus expressed an exceptionally strong (660% ± 21 to 430% ± 18) chemoattractant potency in a narrow (10^{-12}–10^{-11} M) concentration range. Above and below these concentrations, the effect of the peptide was neutral (10^{-16}–10^{-13} M) or mildly chemorepellent (10^{-8}–10^{-6} M; 25–60%). The chemoattractant ability of peptide SEWS compared to the positive controls (f-MLF and IL-8) in the same concentration range shows, however, that these compounds elicit significant chemotactic responses (maximal effect for f-MLF was 212% at 10^{-9} M and 150% for IL-8 at 10^{-11} M, data not shown). SEWS induced chemoattraction with about 3–4 times higher effectiveness even at lower concentrations (10^{-12}–10^{-11} M). In contrast to the pronounced effects described above, the amide of peptide SEWS could not elicit any chemotactic activity in the concentration rage tested (Figure 2b). Thus the amidation of the carboxy terminus results in a significant loss of chemoattractant ability at the low concentrations, however the chemorepellent character observed at higher concentrations was also diminished.

Modification of the peptide sequence on the N-terminus by elongation with a Trp residue (WSEWS) resulted in marked changes in the chemotactic potency. This peptide became chemorepellent (73–85%) at the lower concentrations (10^{-12}–10^{-8} M) (Figure 3a). Substitution at the C-terminal carboxy moiety with an amide group resulted in no changes in the chemorepellent character (57–73%) in the low concentration range (10^{-11}–10^{-9} M) but statistically significant increase in chemotactic index was observed in the relatively high (10^{-8}M, 136%) concentration. This is indicative of the chemoattractant character of the amidated WSEWS ligand (Figure 3b).

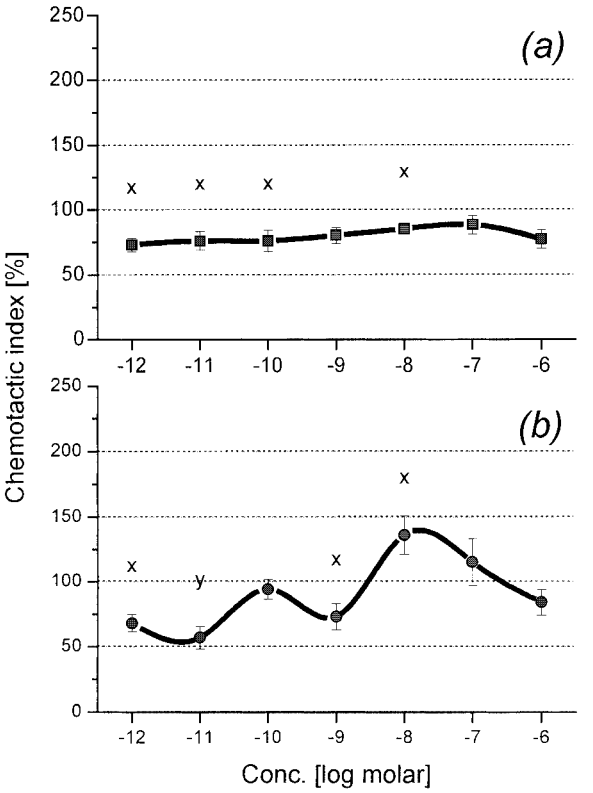

Figure 3. Dose–response correlation of chemosensory responses elicited in *Tetrahymena pyriformis GL* with peptide WSEWS (a) and its amide (b). x, $p < 0.05$; y, $p < 0.01$; z, $p < 0.001$

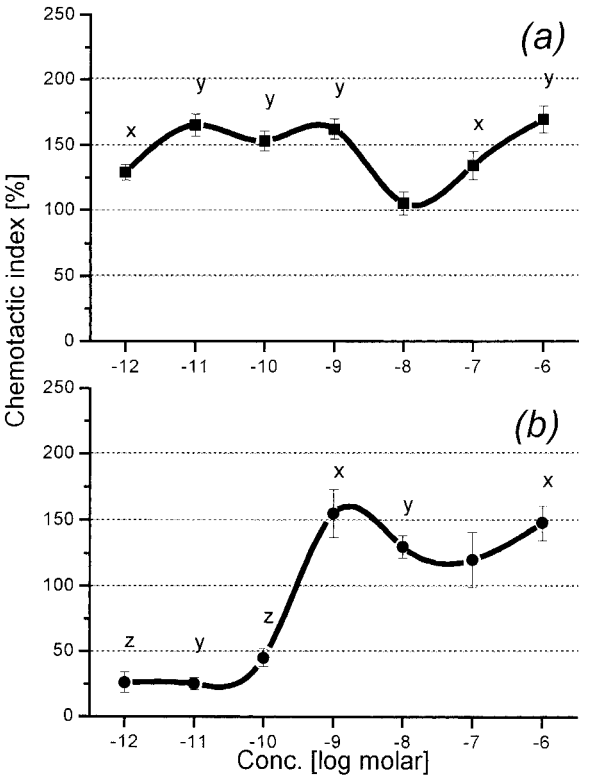

Figure 4. Dose–response correlation of chemosensory responses elicited in *Tetrahymena pyriformis GL* with peptide EWSEWS (a) and its peptide amide (b). x, $p < 0.05$; y, $p < 0.01$; z, $p < 0.001$

Further elongation of the peptide chain at the N-terminus with a Glu residue (EWSEWS) resulted in a peptide with unique. double-peak pattern of concentration-dependent chemotactic activity. Peptide EWSEWS exhibited biologically significant (165–153%) activity in a relatively low, 10^{-11}–10^{-9} M concentration range, while it was also chemoattractant (169%) in the highest (10^{-6} M) concentration tested (Figure 4a). The presence of an amide group at the C-terminus (Figure 4b) resulted in an increased chemotactic index (155–120%) registered in the 10^{-9}–10^{-6} M concentration domain and in decreased values (26–45%) in the lower concentration range (10^{-12}–10^{-10} M). This shows that EWSEWS-amide is chemoattractant for *Tetrahymena* at higher, while chemorepellent at lower concentrations.

The longest oligopeptide tested was GEWSEWS with Gly on the N-terminus. In the case of the heptapeptide a narrow chemoattractant peak was detected at 10^{-10} M (195%). A continuous mild but significant attractant effect could also be observed below (10^{-12}–10^{-11} M; 123–133%; Figure 5a). Amidation of this peptide resulted in the loss of chemoattractant character (Figure 5b) and even provoked some chemorepellent activity at higher concentrations (55–71% at 10^{-9}, 10^{-8}, 10^{-6} M). It is interesting to note that the chemoattractant feature of this elongated peptide amide compared to EWSEWS or WSEWS also expressed a significant conversion to chemorepellent character in a wide concentration range (10^{-10}–10^{-6} M; Figures 2b, 3b and 4b).

DISCUSSION

Chemical characteristics have proved to be decisive in the case of some chemoattractant ligands such as amino acids or bacterial peptides.[26] Previous studies of relatively small oligopeptides showed that chemical modification (e.g. acetylation or methylation) of these peptides can result in a significant loss of chemotactic potency. In this respect terminal parts of the oligopeptides proved to be more significant: modification of the N-terminus of the classical chemoattractant formyl tripeptides to a free amino moiety,

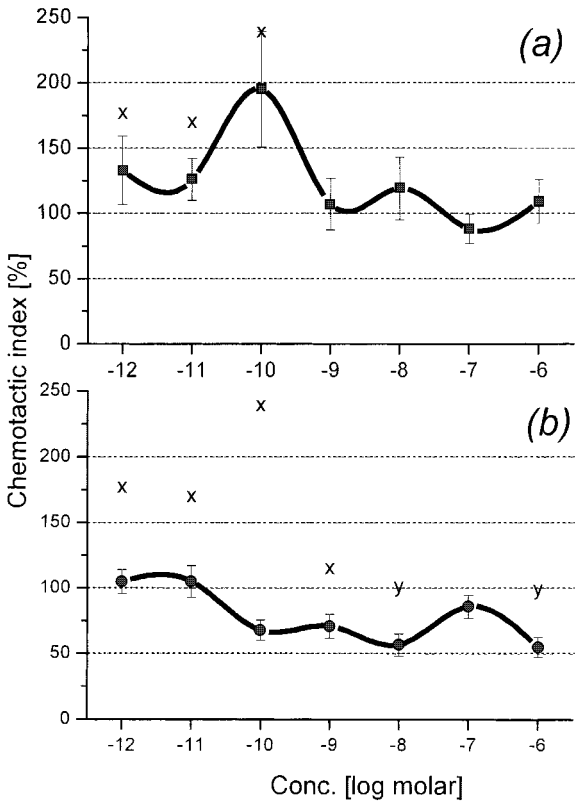

Figure 5. Dose–response correlation of chemosensory responses elicited in *Tetrahymena pyriformis GL* with peptide GEWSEWS (a) and its peptide amide (b). x, $p < 0.05$; y, $p < 0.01$; z, $p < 0.001$

acetyl derivative or desamino analogue results in less active peptides than the parent peptide.[27] However, alteration of the N-terminal amino acid to a more lipophilic one may produce more chemoattractant derivatives in some dipeptides.[5] Model experiments with relatively short peptides e.g. formyl peptides[27] or proline-containing dipeptides[5] indicated that the role of the C-terminus in chemotactic ligands is unclear. In the case of longer ligands composed of the same amino acid residues (e.g. Gly, Ala or Lys) there are direct, positive correlations between the length of the peptide chains and their chemotactic or binding characteristics.[28] This obscure role of the C-terminus motivated the present studies to evaluate the significance of amidation of the C-terminus in four, systematically elongated and biologically significant derivatives, of the EWS ligand.

Our data presented in this paper show that elongation of the native peptide or its amide variants induces divergent alterations concerning the chemotactic ability of the ligands. In the case of the peptides with a free COOH at their C-terminus it should be noted that three peptides of the four were chemoattractant, but the peptide with a WSEWS sequence, which plays a crucial role in the superfamily of cytokine receptors,[29] was neutral at all concentrations tested. This lack of activity may have special significance in understanding the chemorepellent effect of IL-6.[30] Our data described here raise the possibility that the WSEWS motif may also function as a characteristic sequence of soluble cytokine receptor–ligand compleyes. Here, we have to underline the significance of the extreme chemoattractant activity of the SEWS peptide. This potency is present, but at more moderate levels in peptides EWSEWS and GEWSEWS, whereas the shorter or longer derivatives (EWS and WSEWS) have more repellent than neutral character. These observations suggest the possibility that the change in efficacy after modification of SEWS by the removal of S or by elongation with W could be assigned to steric effects. In this regard the significance of Glu (E) can be discussed, because of all the relevant amino acids. Glu has the highest probability (0.93) of having a solvent-exposed area that is larger than 30 sqÅ in peptides.[31] This character of Glu bestows special features to this residue especially in our peptides, where the same index calculated for the neighbouring residues is significantly lower (Ser, 0.70; Trp, 0.49; Gly 0.51). Therefore differences in solvent-exposed areas, and the significance of the negatively-charged side chain of Glu resulting in diversities of the three-dimensional structure, may be responsible for alterations of chemoattractant character of the native peptides.

The present data concerning the significance of amidation supported previous observations. Namely, the carboxy terminal of short peptides proved to be highly variable concerning the structure–fnction relationship in chemotaxis. Our data show that the chemoattractant potency of the tri- and tetrapeptide (EWS and SEWS) shows a strong correlation with the chemical condition of the C-terminus: amidation could enhance the chemotattractant potency (in EWS), but in the one amino acid longer form, SEWS, the amidation (with a parallel elongation of the peptide) could neutralize the highly chemoattractant potency. From comparison of chemotactic potency with the other peptide amides one can conclude that elongation at the N-terminus may influence the *in situ* three-dimensional structure, polarity, charges or size of the solvent-exposed area of the active part (SEWS) of the ligand and amidation results in a set of ligands with variable properties. We found that amidation caused negative (neutral or chemorepellent) effects

both in relatively short (SEWS) and longer (GEWSEWS) derivatives, but also influenced the chemotactic potencies in a positive manner (EWS, WSEWS, EWSEWS).

The data summarized in this communication clearly indicate that cells such as *Tetrahymena* representing a phylogenetically lower level than the frequently used chemotaxis models, *Dictyostelium* or PMN cells, can distinguish slight differences in chemical structure (e.g. amidation, N-terminal elongation) of the ligand even at very low concentrations. High chemotactic responsiveness and the above-mentioned discriminative capacity of *Tetrahymena* support our former theory about the phylogenetically well-conserved signalling mechanisms especially in chemotaxis.[32] Considering this sensitivity and the marked positive responses of this protozoon to the two well-known reference chemoattractants of vertebrates (IL-8 and f-MLF), prompts us to investigate further the SEWS peptide family, especially the highly chemoattractant SEWS in vertebrate systems.

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