A new class of chemotactic peptides containing EWS motif: A mini-review

ESZTER ILLYÉS [1]
SZILVIA BÔSZE [2]
ORSOLYA LÁNG [3]
FERENC SEBESTYÉN [1]
LÁSZLÓ KÔHIDAI [3]
FERENC HUDECZ [2] *

1. Department of Organic Chemistry, Eötvös Loránd University P.O.Box 32 H-1518 Budapest 112, Hungary

2. Research Group of Peptide Chemistry, Hungarian Academy of Sciences Eötvös L. University Budapest, Hungary

3. Department of Genetics, Cell and Immunobiology, Semmelweis University of Medicine, Budapest, Hungary

Corresponding author

ABSTRACT

In this paper we describe the solid phase synthesis and chemical characterisation of oligopeptides corresponding to the IL-6 receptor (EWS, SEWS, WSEWS, EWSEWS and GEWSEWS) and two 19 member libraries (SXWS and WSXWS). Comparative analysis of the chemotactic properties of these ompounds resulted in the identification of a highly active chemoattractant, peptide SEWS, which exhibits a rominent effect (660 ± 21%) at 10^{-12} M on a ciliated protozoon, Tetrahymena pyriformis. *We found that the chemotaxis of cells induced with the SXWS and WSXWS peptides varied according to the nature of the amino acid residue (Ala, Asp, Lys) in position X. Ligand-binding experiments with N-terminally labelled fluorescent derivatives of SXWS peptides, applying [4-[7-hydroxycoumaryl]]acetic acid (Hca-OH) or 4-ethoxymethylene-2-[1]-naphthyl-5(4H)-oxazolone (naOx-OEt) showed good correlation between binding profiles and chemotactic responsiveness (SEWS >SAWS > SDWS ~ SKWS). Chemotactic responsiveness and the binding characteristics data indicate hat chemosensory activity is a surface-membrane associated event, and it is based on receptor-mediated signalling mechanisms.*

INTRODUCTION

The chemotactic response is considered to be one of the basic physiological activities of living cells. Its phylogenetical background is based on the early phases of ligand-receptor interactions when simple substances consumed as nourishment and special ligands possessing more effects on the metabolic processes were selected in a parallel process of the ligand and its receptor (1). Chemotactic receptors are sensitive to slight structural changes of the ligand. For example they can distinguish D-L isomers of amino acids (2), or oligopeptides with or without N-terminal formyl group (3). However, our knowledge concerning the structure-function relationship of such ligands is still far from complete.

Ciliated protozoa (e.g. *Tetrahymena sp.*) have been established models for investigation of chemotaxis and other biological phenomena for some 70 years (4,5). Various oligopeptides including formyl-tripeptide fMLF (6) possess chemoattractant activity towards this type of unicellular organism (7). The membrane receptor composition of unicellular eukaryotic ciliate *Tetrahymena pyriformis* (e.g., insulin (8) or opiate receptors (9)), second messenger systems (e.g., cAMP (10), IP_3 (11) or Ca^{2+}-calmodulin (12)) and intracellular metabolic pathways (13) show close homologies to higher ranked animals. This well characterised protozoan model provided us a suitable experimental tool to establish structure-function relationship in a new class of oligopeptides containing EWS motif. These data can then be utilised to gain new results concerning phylogenetical backgrounds of signalling in chemotaxis.

In a systematic study (14) we have investigated the chemotactic properties of 3 to 7-mer oligopeptides and the corresponding tetra and pentapeptide libraries derived from the sequence of one of the extracellular domains of the interleukin-6 receptor (15). Significance of the EWS-motive selected in biology and especially in immune responses is underlined by the observations that segments of the membrane-bound receptor might induce the cell-surface linked form of migration, haptotaxis (16), while the soluble forms of IL-6 receptors can induce migration of cells in the fluid phase.

In our studies first we have analysed the chemotactic properties of oligopeptides covering the sequence of GEWSEWS. We found that depending on the length of the oligopeptides (EWS, SEWS, WSEWS, EWSEWS and GEWSEWS), all these compounds could have significant chemoattractant effect on *Tetrahymena pyriformis*. In the second phase of our investigation we have compared the chemotactic potency of tetra- and penta-peptide libraries corresponding to the SXWS and WSXWS sequences, respectively and identified SEWS as the most potent variant with the capability to induce a 660 ± 21%

increase in chemotaxis of the cells at 10^{-12} M concentration (17,18). Based on these comparative analyses we have selected four tetrapeptides with different amino acid at position X (Ala, Glu, Asp, Lys) for detailed binding and functional analysis.

MATERIALS AND METHODS

Materials

p-Alkoxybenzylalkohol resin (Wang-resin, 0.96 mmol/g) was obtained from Bachem, Bubendorf, Switzerland (Cat. No. D 1250; lot 504143). AM-RAM resin (0.81 mmol/g) was a product of Rapp Polymere, Tübingen, Germany. Boc-Ser(Bzl)-resin ester (0.8 mmol/g) was prepared according to Horiki *et al.* (19). Fmoc-L-amino acids (Fmoc-Ala, Fmoc-Asp(O^{t}Bu), Fmoc-Glu(O^{t}Bu), Fmoc-Lys(Boc), Fmoc-Ser(^{t}Bu), Fmoc-Trp) as well as Boc-L-amino acids (Boc-Arg(Tos), Boc-Asn, Boc-Gln, Boc-Gly, Boc-His(Tos), Boc-Ile, Boc-LeuH_2O, Boc-Met, Boc-Phe, Boc-Pro, Boc-Ser(Bzl), Boc-Thr(Bzl), Boc-Trp, Boc-Tyr(Bzl), Boc-Val) were purchased from Fluka AG, Buchs, Switzerland. Reagents (DIC, DMAP, EDIA, HOBt, piperidine, TFA) and solvents (DMF and MeOH) were Fluka products of analytical grade. [4-[7-Hydroxycoumaryl]]acetic acid (*Hca*-OH) was prepared according to Baker *et al.* (20), 4-ethoxymethylene-2-[1]-naphthyl-5(4H)-oxazolone (*naOx*-OEt) was synthesized by the method of Kóczán *et al.* (21).

Synthesis

Synthesis of peptides and peptide amides derived from GEWSEWS sequence

The coupling of Fmoc-Ser(^{t}Bu) to the Wang resin was performed with DIC using DMAP as acylating catalyst (22). Wang resin (1 g, 0.96 mmol) was swollen in 10 ml DMF then Fmoc-Ser(^{t}Bu) (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, washed with DMF, EtOH and DMF. The whole coupling procedure was then repeated.

In typical experiments 50-100 mg of Fmoc-Ser(^{t}Bu)-Wang resin (35-70 µmol) or 50-100 mg of Fmoc-Ser(^{t}Bu)-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 x 2 min). 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 x 1 min), MeOH (2 x 1 min) and DMF (4 x 1 min). In the course of coupling of the next amino acid residue, 200% molar excess of Fmoc-amino acid and that of HOBt (23) and DIC (24) 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 x 2 min) and MeOH (2 x 2 min). The conversion was monitored with ninhydrin test (25). After a final deprotection step followed by washing and drying, 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, centrifuged in a sealed tube. The precipitate washed three times with ether, dissolved in water or diluted AcOH and freeze dried.

Parallel synthesis of SXWS and WSXWS peptide libraries

X corresponded to one of 19 proteinogen amino acids (Cys was omitted). Four SXWS peptides (SAWS, SDWS, SEWS and SKWS) were synthesized using Fmoc/^{t}Bu method (see the preceding paragraph). The other 15 tetrapeptides and WSXWS peptides were prepared by Boc/Bzl technique (26,27) as follows.

Boc-Ser(Bzl)-resin (0.1 mmol; ca. 125 mg) was swollen in DCM, deprotected with a mixture of TFA and DCM (1:2, v/v; 2 and 30 min) then washed with DCM, EtOH, DCM, EtOH, 2 min each. Deprotonation was performed with EDIA/DCM mixture (1:9, v/v; 3 x 3 ml, 3 x 5 min), followed by washing with DCM (3 x 2 min). Three-fold molar excess of activated Boc-amino acids (HOBt/DIC) and 2 ml of DCM/DMF solvent mixture (2:1, v/v) was used, reaction time being 120 min. After a final deprotecting, washing and drying, peptide was cleaved from the resin (100 mg) by stirring with 3 ml of a TFMSA/TFA mixture (1:9, v/v) (28) and 20 mg of indole at RT, for 60 min (in the case of peptides containing Arg, 120 min). The resin was removed by filtration, the filtrate was mixed with 25 ml of dry ether. The precipitate was filtered, washed with ether, dissolved in water and neutralized, under cooling, with sodium hydroxide. The solution was freeze dried and the crude peptide was purified with HPLC.

Labelling of Peptides

The fluorophore moiety was introduced on to the SXWS sequence either by reacting the *N*-deprotected resin-bound peptide or the free peptide in solution with the appropriate reagent (17). *N*-terminal labelling of resin-bound peptide with *Hca*-OH was carried out as a normal coupling cycle using the fluorescent carboxylic acid instead of an Fmoc-amino acid (29). The coupling was repeated to achieve complete acylation. For the reaction of *N*-terminus with *naOx*-OEt (21) the fluorescent reagent was dissolved in DMF (2-3 ml) and the resin was shaken for 30 min. It was then washed with DMF and MeOH. Coupling of oligopeptides with fluorophore reagent was performed in solution by adding *Hca*-OH in the presence of DIC and HOBt (1:1:1 mol/mol/mol) or *naOx*-OEt in the presence of EDIA to HPLC purified oligopeptide in DMF. The mixture allowed to stand for 1 h at room temperature, diluted with water, evaporated *in vacuo*; the oily residue diluted and evaporated again, and purified by HPLC.

RP-HPLC

Analytical experiments were made on a Phenomenex Jupiter C18 (250 x 4.6 mm) column (300 Å, 5 µm) or on a DeltaPak C18 (300 x 3.9 mm) column

(300 Å, 15 μm) with a flow rate of 1.0 ml/min. The peptides were purified by using a semi-preparative Phenomenex Jupiter C18 (250 x 10.0 mm) column (300 Å, 10 μm) or DeltaPak C18 (300 x 15.0 mm) column (300 Å, 15 μm) with flow rate of 4.0 ml/min. A Waters HPLC system composed of No. 600 pump, No. 600 controller and No. 490 programmable multiwave length detector was used, with a linear gradient of 80% acetonitrile in 0.1% aqueous TFA.

Mass Spectrometry

Mass spectra (ESI) were recorded on a Perkin Elmer Sciex API2000 or a Finnigan MAT 95SQ tandem mass spectrometer equipped with an ionspray source. Samples were dissolved in a mixture of MeOH and water (1:1) containing 0.05% AcOH. 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 matrix before subjected to FAB-MS analysis.

Cells and Culturing

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

Chemotaxis Assay

Chemotactic responsiveness of *Tetrahymena* cells was evaluated in a two-chamber capillary assay (30) as modified by us (31). In this setup, tips of a multi-8-channel automatic pipette served as inner chamber to minimize the standard error of sampling. The wells of the microtitration plates were used as outer chambers. The outer chamber was filled with the cells to be tested, the inner one contained the solution of test peptide with various concentrations (10^{-12}-10^{-6} M). Compounds were dissolved in culturing medium described above. In control experiments, the fresh culture medium was applied as a test substance. After 15 min incubation the samples of inner chambers, containing the chemotactically positive responder cells, were fixed after transferring the content of the inner chambers into PBS (0.05 M phosphate buffer, pH 7.2; 0.9 M NaCl) containing 4% formaldehyde. The number of the cells in samples were evaluated by counting in a Neubauer hemocytometer. The average of ten replica assays of each ligand is presented in the Figures. The statistical analysis was performed by ANOVA of Origin 4.0.

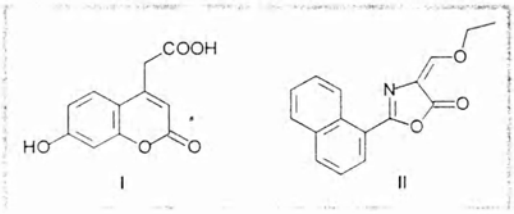
Binding Experiments

For binding experiments *Hca*- or *naOx*-labelled fluorescent SXWS peptides were used. The *Tetrahymena* cells were fixed with PBS containing 4% formaldehyde for 5 min at RT, then washed three times with PBS. The *Hca*- or *naOx*-labelled SXWS derivatives were added in 10^{-6} M concentration to the samples (cell density $5x10^4$ cell/ml), and incubated for 60 min at room temperature. After that, the samples were washed three times with PBS to remove the excess of labelled peptides and the fluorescent activity was measured with a 96 well ELISA plate reader (Labsystems Multiscan MS, using λ_{ex} 340 nm, λ_{em} 460 nm). The average of eight replica assays of each ligand was represented in the figures. The statistical analysis was performed by ANOVA of Origin 4.0.

RESULTS AND DISCUSSION

Solid Phase Synthesis and Characterisation of Peptides, Peptide Amides and Fluorescent Derivatives

In order to analyse the chemotactic properties of oligopeptides corresponding to the GEWSEWS sequence of IL-6 receptor first 3-7-mer peptides with free or amidated C-terminus with EWS motif were prepared. For the second phase of our studies tetra- and penta-peptide libraries were produced with SXWS and WSXWS amino acid sequence, respectively. Finally four peptides from the SXWS library containing the non-charged alanine (SAWS), acidic amino acids (aspartic acid, SDWS; glutamic acid, SEWS) and a basic amino acid (lysine, SKWS) were N-terminally labelled with fluorophores. Considering the potential influence of the *N*-terminal modification on binding properties, two different reagents ([4-[7-hydroxycoumaryl]]acetic acid (**I**) and 4-ethoxymethylene-2-[1]-naphthyl-5(4H)-oxazolone (**II**)) were used for the introduction of fluorophores.



The 3-7-mer peptides and peptide amides containing EWS motif and four members of SXWS library (SAWS, SDWS, SEWS and SKWS) were prepared by solid phase synthesis using the Fmoc/^{t}butyl technique (32,33), while the other 15 SXWS peptides and the WSXWS peptide library were synthesized with Boc/Bzl method (26,27), applying an *in situ* active ester (HOBt/DIC) coupling strategy in parallel format in both techniques. The synthetic protocol is described in the experimental part. The use of Fmoc-Trp and Boc-Trp without any protecing group on the indole ring of their side chain gave satisfactory results. The fluorescent labelling of the *N*-termini of selected tetrapeptides was performed either as the last step of SPPS on resin-bound peptide by reaction with *Hca*-OH (20,29) or *naOx*-OEt (21) or by the

treatment with appropriate reagents with tetrapeptides in solution. The comparison of labelling strategies with *naOx*-derivatives and *Hca*-labelled peptides clearly showed that the coupling performed in solution using HPLC purified oligopeptides results in a crude product with one predominant peak. In contrast, the coupling to partially protected peptides bound to the resin led to a more complex mixture of products after cleavage.

Crude peptide samples were purified by HPLC. The final products were characterised by HPLC retention time values and MS data. The spectroscopic properties of labelled peptides were characterised by measurement of their absorption and emission spectra (34). We found that the absorption maxima of all peptide derivatives, regardless of the presence of *naOx*- or *Hca*-groups, were at λ =340 nm in 0.14 M phosphate buffer, pH=7.4. Under the same conditions the emission maximum for *Hca*-peptides was at λ=466 nm, and that of for *naOx*-derivatives at λ=445-480 nm. These values are in the range of published spectral characteristics of *Hca*-(29) or *naOx*-peptides (21) of different length and composition.

Characteristic data of oligopeptides are presented in Tables I-IV.

Table II - Chemical characterisation of SXWS peptides

Peptide	Relative Molecular Mass Calculated	Observed	R_t (min) [c]	Peptide	Relative Molecular Mass Calculated	Observed	R_t (min) [a]
SAWS	449.2	449.8 [a]	20.1	SNWS	492.2	493.3 [b]	20.0
SDWS	493.2	493.8 [a]	20.2	SPWS	475.2	476.2 [b]	26.4
SFWS	525.2	526.3 [b]	26.4	SRWS	534.2	535.3 [b]	20.9
SGWS	435.2	436.3 [b]	20.7	SSWS	465.2	466.3 [b]	20.9
SHWS	515.2	516.1 [b]	23.8	STWS	479.2	480.4 [b]	20.0
SIWS	491.2	492.1 [b]	26.1	SVWS	477.2	478.3 [b]	24.6
SKWS	506.2	506.9 [a]	19.3	SWWS	564.2	565.3 [b]	29.0
SLWS	491.2	492.4 [b]	25.4	SYWS	541.2	542.2 [b]	24.7
SMWS	509.2	510.2 [b]	26.5				

Relative molar mass was determined by a. ES or b. FAB MS

c. HPLC was performed on a Phenomenex Jupiter C18 (250 x 4.6 mm) column (300 Å, 5 µm) with a flow rate of 1.0 ml/min at RT, detection at λ = 220 and 280 nm. Eluents: A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile/water (80:20, v/v). B 5 to 55% in 25 min;

Chemotaxis Induced by 3-7-mer EWS-Peptides with Free or Amidated C-Terminus

Comparison of chemotactic effects of peptides summarised in Table I was performed to identify active peptides within the set of oligomers containing EWS motif and to analyse the effect of N-terminal elongation (14). We have compared the activity of peptide acid with that of peptide amide under identical experimental conditions to gain better understanding on the influence of amidation on chemotactic activity.

The shortest peptide EWS with carboxyl group at its C-terminus showed a strong chemorepellent activity (50-75%) in a wide concentration range (10^{-10}-10^{-6} M), while the amidated variant was chemoattractant at low (140-150%, 10^{-12}- 10^{-10} M) and also at high concentrations (210-225%, 10^{-9}-10^{-7} M). In the case of SEWS peptide with COOH at the C-terminus expressed an exceptionally strong (660% ± 21 - 430% ± 18) chemoattractant potency in a narrow (10^{-12}-10^{-11} M) concentration range. Over and below these concentrations the effect of the peptide was neutral (10^{-16}-10^{-13} M) or mildly chemorepellent (10^{-8}-10^{-6} M). The amidation of the carboxy-terminus results in a significant loss of both chemoattractant and chemorepellent character.

Elongation of the peptide sequence on the N-terminus by a Trp residue (WSEWS) resulted in marked changes in the chemotactic potency. This peptide turned to be chemorepellent (75% ± 5) in the lower concentrations (10^{-12}-10^{-8} M). Introduction of amide moiety at the C-terminal resulted in no changes in the chemorepellent character in the low concentration range (10^{-11}-10^{-9} M). Further elongation of the peptide chain at the N-terminus resulted in a peptide with unique pattern of concentration dependent chemotactic activity.

Peptide EWSEWS exhibited significant chemoattractant activity in a relatively low, 10^{-12}-10^{-9} M concentration range (125-170%). EWSEWS-amide was chemoattractant for *Tetrahymena* at higher concentrations (10^{-9}-10^{-6} M, 125-160%), while chemorepellent at lower concentration range (10^{-12}-10^{-10} M, 25-50%). The longest oligopeptide (GEWSEWS) tested has a chemoattractant peak at c=10^{-10} M (200%). Amidation of this peptide resulted in the loss of

Table I - Chemical characterization of peptides and peptide amides with EWS motif

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

a. Relative molar mass was determined by FAB mass spectrometry

b. HPLC was performed on a C18 DeltaPak 300 Å, 15 µm, 300 x 3.9 mm column, with a flow rate of 1.0 ml/min at RT, detection at 220 nm. Eluents: A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile/water (80:20, v/v)

c. B 1-55% in 25 min;

d. B 15-45% in 30 min;

e. B 5-55%

Table III - Chemical characterisation of WSXWS peptides

Peptide	Relative Molecular Mass Calculated	Observed	R_t (min) [c]	Peptide	Relative Molecular Mass Calculated	Observed	R_t (min) [a]
WSAWS	635,3	635,9 [a]	25,3	WSNWS	678,3	679,3 [b]	25,2
WSDWS	679,2	679,9 [a]	25,2	WSPWS	661,3	662,4 [b]	27,2
WSFWS	711,3	712,3 [b]	31,0	WSRWS	720,3	721,3 [b]	24,0
WSGWS	621,2	622,5 [b]	25,4	WSSWS	651,2	652,3 [b]	26,2
WSHWS	701,3	702,2 [b]	25,5	WSTWS	665,3	666,3 [b]	26,3
WSIWS	677,3	678,4 [b]	30,0	WSVWS	663,3	664,5 [b]	28,3
WSKWS	692,3	692,9 [a]	24,6	WSWWS	750,3	751,3 [b]	31,1
WSLWS	677,3	678,5 [b]	30,5	WSYWS	727,3	728,3 [b]	27,2
WSMWS	695,3	696,3 [b]	27,5				

Relative molar mass was determined by a. ES or b. FAB MS

c. HPLC was performed on a Phenomenex Jupiter C18 (250 x 4.6 mm) column (300 Å, 5 μm) with a flow rate of 1.0 ml/min at RT, detection at λ = 220 and 280 nm. Eluents: A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile/water (80:20, v/v). B 5 to 55% in 25 min;

Chemotaxis of SXWS- and WSXWS-Peptides

In order to analyse the effect of the amino acid side chain in position X of SXWS and of WSXWS sequences on the chemoattraction two peptide libraries were prepared by parallel SPPS (Tables II and III).

Chemotactic response of each library containing 19 tetra- or penta-peptides was evaluated in a two-chamber capillary chemotaxis assay using conditions defined above in a wide concentration range. The chemosensory activity of peptides was compared at concentration where the maximum response was observed. Results of these studies are expressed as chemotaxis index values and presented in Figures 1 and 2 as a function

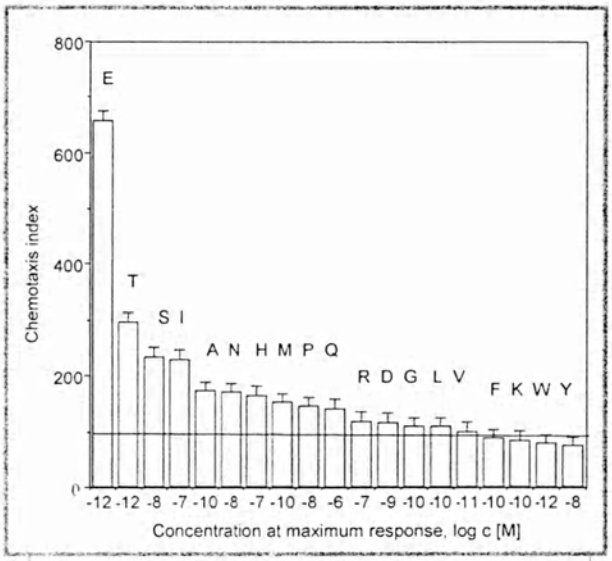


Figure 1 - SXWS peptides induced chemosensory responses measured at concentration at maximum response in *Tetrahymena pyriformis*

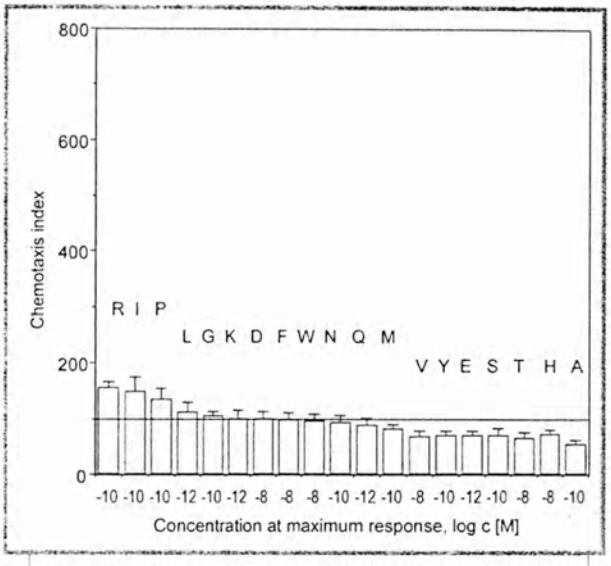


Figure 2 - WSXWS peptides induced chemosensory responses measured at concentration at maximum response in *Tetrahymena pyriformis*

chemoattractant character and even provoked some chemorepellent activity at higher concentrations (50-75% at 10^{-10}-10^{-6} M).

Taken together the analysis of the synthetic 3-7-mer IL-6 receptor peptides showed that SEWS possesses exceptionally strong chemoattractant ability. Conspicuously, the shorter peptide (EWS) and the longer one (WSEWS) have chemorepellent activity, while EWSEWS and GEWSEWS displayed no characteristic chemotactic properties. Amidation of C-terminus significantly modified the character of peptides: in contrast to the repellent activity of EWS, its amide is definitely attractant. While SEWS was the most active chemoattractant, its amide proved to be chemotactically inactive. Based on these findings it seems that both size and polarity of the peptides are important in the nature of their chemotactic character.

of peptide concentration. Data on Figure 1 showed that SEWS had the strongest chemoattractant activity. Nine peptides (STWS, SSWS, SIWS, SAWS, SNWS, SHWS, SMWS, SPWS and SQWS) were moderately chemoattractant (chemotaxis index: 300 and 150%). No significant chemotactic effect was observed in the case of SRWS, SDWS, SGWS, SLWS, SVWS, SFWS. Three peptides (SKWS, SWWS, SYWS) exhibited some repellent character (chemotaxis index: 90 and 75%).

Peptides corresponding to the WSXWS sequence display a lower level of diversity in inducing chemotactic activity (Figure 2). Three peptides (WSRWS, WSIWS and WSPWS) can be considered as chemoattractant with moderate chemotaxis index values (150-130%). Six peptides, where X = L, G, K, D, F or W were inactive in this assay, while the

majority of pentamers tested proved to possess moderate, but statistically significant chemorepellent character (95-50%).

Although it is difficult to draw conclusions from these two sets of data without published results on the receptor structure involved it is interesting to detect changes caused by the addition of Trp residue to the SXWS sequence. Comparison of the most active peptides, SEWS, STWS, SSWS and SIWS with respective WSXWS peptides it is clear that the presence of N-terminal Trp resulted in dramatic reduction of the chemotaxis index values of three out of four compounds (SEWS vs. WSEWS, STWS vs. WSTWS and SSWS vs. WSSWS). On the other hand the chemorepellent feature of SYWS was essentially not changed by the introduction of Trp in WSYWS. These data might indicate that besides the side chain structure of amino acid X, the identity of the N-terminal amino acid residue, and also the length of the peptide chain could play an important role in the induction of chemosensory response in *Tetrahymena*.

Comparison of Chemotactic Properties and Binding of Selected SXWS Peptides

Based on the results obtained from the 19 tetra- and penta-peptide libraries four peptides containing the highly chemoattractant SEWS, were selected for detailed studies. In these compounds amino acid with non-charged aliphatic (alanine, SAWS), with negatively charged (aspartic acid, SDWS) or with positively charged (lysine, SKWS) side chain is located at the X amino acid position. The analysis of chemoattractant/chemorepellent properties of SAWS, SDWS and SKWS was extended by the study their cell-binding properties and of chemotactic selection (17). For the latter experiments, two sets of fluorescent derivatives of peptides were prepared.

The concentration dependent chemotaxis (Figure 3) in accord with previous results indicated that four tested tetrapeptides elicited different, but

Figure 3 - Dose-response correlation of chemosensory responses induced by SXWS peptides in *Tetrahymena pyriformis*

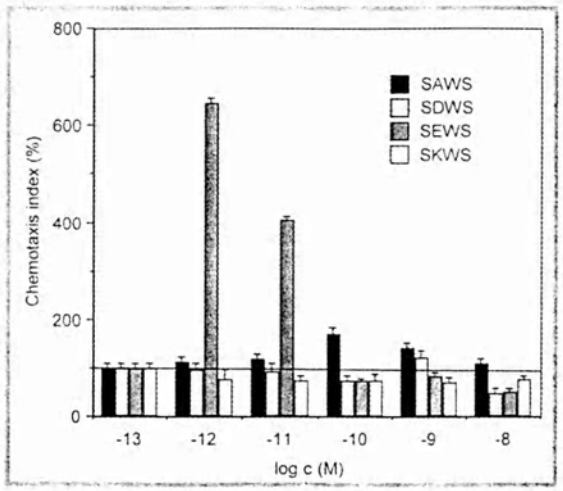


Table IV - Chemical characterisation of fluorescent SXWS peptides

Peptide and peptide derivative	Relative Molecular Mass [a] Calculated	MH+ observed	R_t [b]
Hca-SAWS	651.2	652.0	25.2 [c]
Hca-SDWS	695.2	696.1	25.0 [c]
Hca-SEWS	709.2	710.2	24.6 [c]
Hca-SKWS	708.2	709.0	24.0 [c]
naOx-SAWS	670.2	670.9	37.0 [d]
naOx-SDWS	714.2	714.7	35.5 [d]
naOx-SEWS	728.2	728.5	34.8 [d]
naOx-SKWS	727.3	728.2	41.1 [d]

a. Relative molar mass was determined by ES mass spectrometry
b. HPLC was performed on a Phenomenex Jupiter C18 (250 x 4.6 mm) column (300 Å, 5 µm) with a flow rate of 1.0 ml/min at RT, detection at λ = 220, 280 and 360 nm. Eluents: A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile/water (80:20, v/v)
c. B 5 to 55% in 25 min
d. B 20 to 70% in 45 min
e. B 20 to 90% in 60 min.

characteristic, chemotactic responses. Peptide SAWS was chemoattractant in a narrow concentration range (10^{-10}-10^{-9} M): the maximal effect was 174 ± 20% at 10^{-10} M. Peptide SKWS, however, showed small but statistically significant (80-83%) chemorepellent activity over a wide concentration range (10^{-11}-10^{-8} M). The chemotactic properties of SXWS peptides containing acidic amino acid at position X (SEWS and SDWS) were found to be very much dependent on the side chain of the amino acid X. The peptide SEWS induced an intense chemoattractant response in a low and narrow concentration range peaking with 660 ± 21% at c=10^{-12} M. At a higher peptide concentration (c=10^{-8}-10^{-6} M), this compound exhibited significant chemorepellent character (25-70%). In sharp contrast, peptide SDWS, also with COOH in the side chain did not elicit any significant chemotactic response of *Tetrahymena* cells. Interestingly the chemotactic responses elicited by the respective free amino acids (Glu and Asp) showed similar tendency (17).

The chemoattractant ability of peptide SEWS was compared with compounds eliciting significant chemotectic responses in the same concentration range. The maximal effect of f-MLF and IL-8 used as positive control was 212% at 10^{-9} M for f-MLF and 150% for IL-8 at 10^{-11} M. These data emphasize the exceptional capability of the newly found peptide, SEWS in induction of chemoattraction.

Chemotaxis is a membrane receptor linked process in prokaryotes and eukaryotes alike. The integrity of these structures in the surface membrane (*e.g.*, aspartate (35) or dipeptide receptors of *E. coli* and fMLF or chemokine receptors of PMN cells (36,37)) is responsible for the specificity of chemotactic responses.

Considering this receptor-mediated chemotactic

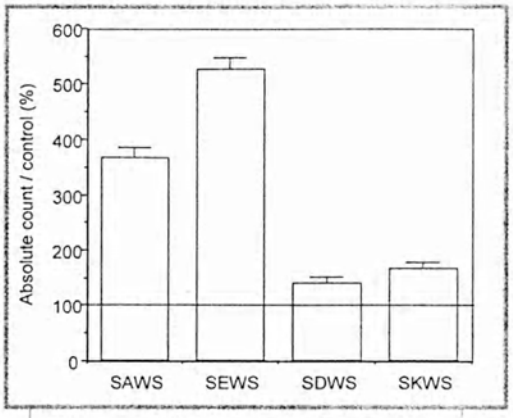


Figure 4 -Binding of naOx- labelled SXWS peptides to fixed *Tetrahymena pyriformis* cells

signalling, the detection of ligand binding properties of target cells is important, complementary measures of functional studies of chemotaxis. For binding experiments, we have utilised two new groups of SXWS peptides labelled with *Hca*- or *naOx*-fluorophore (Table IV).

The binding of labelled peptides to fixed *Tetrahymena* cells was studied by measuring the fluorescent activity of cell samples. Based on values expressed as % binding compared to the control (100%), we found that both *Hca*- and *naOx*-modified tetrapeptides bound to cell membrane in different degree. The binding of *naOx*-SXWS peptides showed significantly higher activity. This might be due to the composition and structure of the two chromophores. The binding characteristics of fixed *Tetrahymena* cells with the *naOx*-labelled peptides showed a good correlation with the chemotactic responsiveness of sub-populations selected by the identical peptides (SEWS > SAWS > SDWS ~ SKWS) (Figure 4).

The results of binding assays gained with *naOx*- and *Hca*-labelled SXWS peptides and the detected functional activity of *Tetrahymena* suggest that the studied chemotactic response is based on a receptor-linked signalling pathway.

CONCLUSIONS

Our data suggest that in the groups of synthetic peptides, WSXWS and SXWS derived from the GEWSEWS sequence of vertebrate IL-6 receptor also active in a simple phylogenetically distant eukaryote unicellular organisms, *e.g.*, *Tetrahymena pyriformis*. Using this model system we found that the nature of X residue in WSXWS and SXWS peptides influences markedly the chemoattractant/chemorepellent effect on *Tetrahymena pyriformis* cells. The concordant results of chemotactic responsiveness elicited by the peptides (SAWS, SEWS, SDWS) and their binding characteristics indicate that chemotactic activity is a surface-membrane associated event, and it is based on receptor-mediated signalling mechanisms.

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