

Identification of SNARE complex modulators that inhibit exocytosis from an α -helix-constrained combinatorial library

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Synthetic peptides patterned after the proteins involved in vesicle fusion [the so-called SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins] are potent inhibitors of SNARE complex assembly and neuronal exocytosis. It is noteworthy that the identification of peptide sequences not related to the SNARE proteins has not been accomplished yet; this is due, in part, to the structural constraints and the specificity of the protein interactions that govern the formation of the SNARE complex. Here we have addressed this question and used a combinatorial approach to identify peptides that modulate the assembly of the SNARE core complex and inhibit neuronal exocytosis. An α -helix-constrained, mixture-based, 17-mer combinatorial peptide library composed of 137 180 sequences was synthesized in a positional scanning format. Peptide mixtures were assayed for their ability to prevent the formation of the *in vitro*-reconstituted SDS-resistant SNARE core complex. Library deconvolution identified eight peptides that

inhibited the assembly of the SNARE core complex. Notably, the most potent 17-mer peptide (acetyl-SAAEAFKLYAEAFKGNH₂) abolished both Ca²⁺-evoked catecholamine secretion from detergent-permeabilized chromaffin cells and L-glutamate release from intact hippocampal primary cultures. Collectively, these findings indicate that amino acid sequences that prevent SNARE complex formation are not restricted to those that mimic domains of SNARE proteins, thus expanding the diversity of molecules that target neuronal exocytosis. Because of the implication of neurosecretion in the aetiology of several human neurological disorders, these newly identified peptides may be considered hits for the development of novel anti-spasmodic drugs.

Key words: combinatorial chemistry, drug discovery, neurosecretion, protein–protein interaction, synaptic transmission, vesicle fusion.

INTRODUCTION

Calcium-regulated exocytosis in excitable cells is mediated by the precise docking and fusion of neurotransmitter-loaded cargo vesicles [1,2]. Mechanistically, neuronal exocytosis is an orchestrated cascade of protein–protein interactions that involve several proteins. At the centre of the process are found the so-called SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins, which include the plasma membrane proteins SNAP25 (synaptosomal-associated protein of 25 kDa) and syntaxin, and the vesicle-associated protein VAMP (vesicle-associated membrane protein; synaptobrevin) [1–3]. These proteins assemble into a highly stable, ternary complex known as the SNARE core complex [1,2,4,5]. Cumulative evidence has shown that this protein complex is responsible for vesicle docking and fusion in synaptic terminals [1,2]. Indeed, interference with the formation or thermal stability of the SNARE complex severely abrogates Ca²⁺-regulated exocytosis at synaptic junctions [1,2].

Structurally, the SNARE complex is a parallel four-helix bundle formed by the coiled-coil arrangement of two helices from SNAP25 and one each from syntaxin and VAMP [4,5]. The centre of the complex shows the presence of leucine-zipper layers

interspersed with an ionic layer and surrounded by a hydrophobic core [4,5]. This structural arrangement endows the core complex with notable thermal and chemical stability, and provides the energy to drive fusion of the lipid bilayers [4–6]. The high stability of the SNARE complex has hampered the discovery of small molecules that modulate the assembly of the proteins. Thus far, clostridial neurotoxins and peptides patterned after protein domains of SNARE proteins have been the only molecules able to modulate the assembly and stability of the core complex [7–19]. Peptides that mimic domains of SNARE proteins act as competitive antagonists [8–19]. The amino acid sequence and length of these peptides is critical for their inhibitory activity [14,15]. These peptides may assemble into a coiled-coil structure akin to that of the SNARE complex [20]. However, the discovery of amino acid sequences unrelated to the SNARE proteins that are capable of inhibiting the assembly of the core complex remains elusive.

To address this issue, we have used a combinatorial approach based on the screening of a peptide library built on an α -helical scaffold. Conformation-constrained libraries have been used to identify antimicrobials and inducers of viral infectivity [21–23]. The rationale for selecting this library considered that the SNARE complex is a four-helix bundle and that peptides that

Abbreviations used: Ac, acetyl; BSS basic saline solution; OG, octyl β -D-glucopyranoside; SNAP25 synaptosomal-associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; VAMP, vesicle-associated membrane protein (synaptobrevin); [O⁶O⁹O¹⁰F¹⁴], shortened peptide nomenclature referring to the amino acids preferred at the combinatorialized positions of the 17-mer (e.g. Ac-SAAEAFKLYAEAFKGNH₂ is referred to as [F⁶L⁹Y¹⁰F¹⁴]).

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prevent its assembly exhibit a high propensity to adopt an α -helical secondary structure [4–6,20]. Screening and deconvolution of the α -helical peptide library identified eight peptides that inhibited the formation of the SDS-resistant SNARE complex with different potencies. The most active 17-mer peptide (Ac-SAAEAFAKLYAEAFKNG-NH₂, where Ac is acetyl) abrogated the Ca²⁺-dependent release of L-[³H]glutamate in intact hippocampal neurons. These findings are a proof-of-principle that peptide modulators of SNARE complex assembly are not restricted to those that mimic domains of the SNARE proteins. Because dysfunctional exocytosis is involved in the aetiology of several human diseases, these peptides may provide scaffolds for the development of novel anti-spasmodic drugs.

EXPERIMENTAL

Synthesis of the peptide library and of individual peptides

The library and synthetic peptides were synthesized by simultaneous multiple-peptide synthesis using Fmoc (fluorenylmethoxycarbonyl) chemistry, as described in [22–24]. The mixture ('X') positions were incorporated by coupling a mixture of 19 L-amino acids (cysteine was omitted), with the relative ratio solubility adjusted to yield close to equimolar incorporation. The quality of the synthesized peptide mixtures was validated by electrospray MS. Individual peptides were purified by preparative reverse-phase HPLC. Peptide identity was confirmed by laser-desorption time-of-flight MS.

Expression and purification of recombinant SNARE proteins

Recombinant bacterially expressed SNARE proteins were obtained as described [25]. Briefly, glutathione S-transferase fusion proteins were expressed in the BL21DE3 *Escherichia coli* strain. Protein expression was induced with 1 mM isopropyl β -D-thiogalactoside for 5 h at 30 °C. Bacterial cultures were pelleted, washed with lysis buffer (10 mM sodium phosphate, pH 7.4, 136 mM NaCl, 2.7 mM KCl), digested with 0.1 mg/ml lysozyme for 10 min at 22 °C in lysis buffer, supplemented with 2 mM PMSF, 5 mM iodoacetamide and 5 mM EDTA, and sonicated (3 \times 45 s) in a Branson 250 sonifier at 4 °C. Lysates were solubilized with 1 % Triton X-100 for 20 min at 4 °C, and cleared by centrifugation at 20 000 g for 30 min at 4 °C. SNAP25 and VAMP were purified from the sonicated bacterial supernatant by affinity chromatography on glutathione-agarose (Pharmacia), following the manufacturer's instructions. Purification of recombinant proteins was carried out in 20 mM Hepes, pH 7.4, 100 mM NaCl, 0.05 % OG (n-octyl β -D-glucopyranoside) and 5 mM dithiothreitol; proteins were cleaved with thrombin for 3 h at 23 °C and then dialysed against 20 mM Hepes, pH 7.0, 80 mM KCl, 20 mM NaCl and 0.1 % OG. Syntaxin was obtained from the bacterial pellet by washing the precipitate with 50 mM Tris/HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl and 1.0 % Triton X-100 in a Polytron (Kinematica). Protein was recovered from inclusion bodies by incubating the solubilized pellet with 50 mM Tris/HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl and 1.0 % N-lauroylsarcosine overnight at 4 °C. Extracted protein was diluted 1:10 (v/v) in washing buffer (10 mM Hepes, pH 7.4, 0.1 % OG) and loaded on to glutathione-agarose resin. Bound material was purified in washing buffer and cleaved with thrombin for 1 h at room temperature. Purified proteins were stored at –80 °C. Concentration was assayed with a BCA kit (Pierce), and purity was verified by SDS/PAGE analysis.

In vitro reconstitution of the SDS-resistant SNARE complex

Syntaxin and VAMP were incubated at final concentrations of 3.0 μ M in the presence/absence of peptides at the indicated concentrations for 1 h. Thereafter, SNAP25 (0.3 μ M) was added and the reactions allowed to proceed overnight. Reactions were carried out at 4 °C in 20 mM Hepes, pH 7.4, 100 mM NaCl, 1.0 % OG and 2.0 mM dithiothreitol in a final volume of 15 μ l, and were stopped by the addition of SDS/PAGE sample buffer. Peptide blockade activity was evaluated on SDS/PAGE polyacrylamide gels (12 %) by monitoring the disappearance of the 75 kDa band corresponding to the ternary core complex. Gels were digitized and quantified as described in [25].

CD spectroscopy

CD was carried out in a JASCO J-810 spectropolarimeter equipped with a computer-controlled temperature cuvette holder. CD data for the far-UV spectra (195–250 nm) were recorded with a 1 mM path-length cell containing 5–20 μ M peptide in 20 mM Tris/HCl, pH 8.0, and 10 % (v/v) acetonitrile. Acetonitrile was added to improve the solubility of the peptides. All spectra were recorded at 25 °C and at 50 nm/min (response time of 1 s), averaged (five scans), and corrected for the buffer contribution. CD signals (in mdegrees) were converted into mean ellipticity (θ ; mdegrees \cdot cm² \cdot dmol⁻¹) using the relationship $\theta_v = 100 \times \text{CD signal} / (C \times N \times l)$, where C denotes the peptide concentration, N the number of residues and l the path length. Secondary structure elements were inferred by fitting the CD spectra [25].

Chromaffin cell cultures

Chromaffin cell cultures were prepared from bovine adrenal glands by collagenase digestion, and were separated further from debris and erythrocytes by centrifugation on Percoll gradients as described [15,16]. Cells were maintained in monolayer cultures at a density of 625 000 cells/cm², and were used between 3 and 6 days after plating. All experiments were performed at 37 °C.

Determination of catecholamine release from detergent-permeabilized chromaffin cells

Secretion of noradrenaline and adrenaline was determined using digitonin-permeabilized cells as described in [15,16]. Briefly, monolayers were washed four times with a Krebs/Hepes basal solution: 15 mM Hepes, pH 7.4, containing 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 0.56 mM ascorbic acid and 11 mM glucose. Cell permeabilization was accomplished with 20 μ M digitonin in 20 mM Pipes, pH 6.8, containing 140 mM monosodium glutamate, 2 mM MgCl₂, 2 mM MgATP and 5 mM EGTA. This incubation was carried out in the absence or the presence of peptides. Following permeabilization, media were discarded and cells were incubated for 10 min in digitonin-free medium in the presence or absence of peptides. Basal secretion was measured in 5 mM EGTA, whereas stimulated secretion was measured in a medium containing 10 μ M buffered Ca²⁺ solution. Media were collected, and released catecholamines as well as the total cell content were determined by HPLC using an electrochemical detector. Statistical significance was calculated using Student's t test with data from four or more independent experiments.

L-[³H]Glutamate release from hippocampal cultures

Mixed hippocampal neuronal/glial cultures were prepared as described [26]. Briefly, hippocampi were dissected from rat embryos (day 17–19) and incubated at 37 °C for 15 min in BSS (basic saline solution, containing in mM: 137 NaCl, 3.5 KCl, 0.4 KH₂PO₄, 0.33 Na₂HPO₄ · 7H₂O, 5 Tes, pH 7.4, and 10 glucose) with 0.025 % (w/v) trypsin. Trypsin was diluted by rinsing the tissue three times for 5 min each with BSS. Tissue was then dissociated by several passes through a siliconized pasteur pipette, first unpolished and then with a fire-polished pipette. Cells were centrifuged for 5 min at 200 g and pellets were resuspended in BSS. Cells were plated at 2 × 10⁵ viable cells/cm² in poly-L-lysine (0.2 mg/ml)-coated dishes. The culture medium had the following composition: minimum essential medium (Earle's salts; GIBCO) supplemented with 10 % (v/v) heat-inactivated horse serum (Sigma), 10 % (v/v) heat-inactivated foetal bovine serum (Sigma), 1 mM glutamine and 22 mM glucose. Cultures were maintained at 37 °C in a 5 % CO₂ atmosphere, and half of the medium was renewed every 2–3 days. At day 5 after plating, glial proliferation was inhibited by the addition of 80 μM 5-fluoro-2'-desoxyuridine.

Neuronal cultures (8–11 days *in vitro*) were used for measuring L-[³H]glutamate release. Hippocampal neurons (3 × 10⁵) were loaded with 2 μCi/ml L-[³H]glutamate in BSS at 37 °C for 90 min. Thereafter, neurons were washed extensively with BSS to remove extracellular L-[³H]glutamate, and incubated with 10 μM peptide at 37 °C for 60 min. L-[³H]Glutamate release was evoked by pulsing the neurons with 56 mM KCl (K⁺-BSS) in the presence of 2.0 mM extracellular Ca²⁺. Ca²⁺-independent L-[³H]glutamate exocytosis was elicited with Ca²⁺-free K⁺-BSS supplemented with 0.5 mM EGTA [27]. Basal release was obtained from unstimulated cultures. Extracellular medium containing the secreted L-[³H]glutamate was harvested, and radioactivity was counted on a β-counter. Values were normalized with respect to the radioactivity of unstimulated neurons (basal).

RESULTS

Design of a conformation-constrained α-helical combinatorial library

A peptide library comprising 17-mer peptides with a high propensity to adopt an α-helical secondary structure was designed using as the scaffold SAAEAXAKXXAEAXAKG, where X denotes the positions that were varied by using an equimolar mixture of L-amino acids (Figure 1A). The fixed and variable positions on the helical scaffold were designed to preserve the tendency of the peptide to fold into an α-helix conformation, and to minimize alternative secondary structures, while allowing sequence diversity. Residues Ser-1 and Gly-17 have N- and C-terminal α-helical, end-capping properties [28,29]. Two positively charged residues, Glu (E; positions 4 and 12) and Lys (K; positions 8 and 16), were incorporated to favour aqueous solubility and the formation of salt bridges that stabilize the helical conformation [30,31]. Alanine residues were chosen because of their intrinsic α-helix-stabilizing properties [32]. The designed scaffold exhibited ≥ 50 % propensity to fold into an α-helical secondary structure, as determined by the AGADIR program [33]. Four positions of the scaffold (residues 6, 9, 10 and 14) were used to provide sequence diversity (Figure 1A). The library was designed in a positional scanning format: Ac-SAAEAOAKXXAEAXAKG-NH₂, Ac-SAAEAXAKOXAEA-XAKG-NH₂, Ac-SAAEAXAKXOAEAXAKG-NH₂ and Ac-SAAEAXAKXXAEAOAKG-NH₂, where O denotes a fixed position

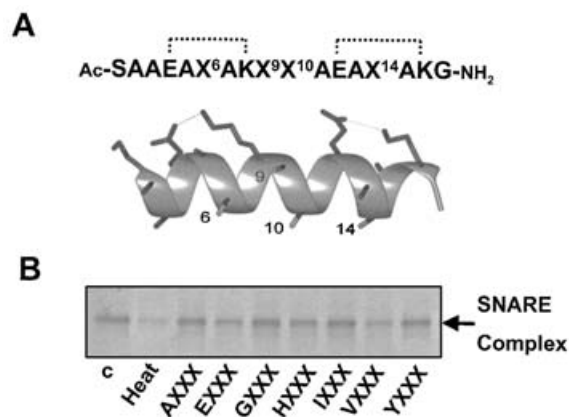


Figure 1 Design of an α-helix-constrained peptide library

(A) Top: peptide scaffold used to create the α-helix-constrained library. A backbone of alanine residues was used. Glutamic acid (positions 4 and 12) and lysine (positions 8 and 16) residues were used to stabilize the helical conformation through the formation of salt bridges. Serine and glycine residues were used as N- and C-caps respectively. Positions 6, 9, 10 and 14 were combinatorialized with a mixture of the naturally occurring L-amino acids (cysteine was omitted). Bottom: helical representation of the library scaffold, highlighting the salt bridges and the combinatorialized positions. (B) An *in vitro*-reconstituted SNARE core complex was used as the molecular target of peptide mixture. The ternary complex was reconstituted *in vitro* by mixing recombinant VAMP, syntaxin and SNAP25 (1:1:0.1 molar ratio). Peptide mixtures were added to a final concentration of 2 mg/ml. SNARE complex formation was evaluated by SDS/PAGE as a band of 75 kDa (arrowed). Disruption of the ternary complex may be followed as a decrease in the intensity of this 75 kDa band. c, control.

defined with one of the 20 L-amino acids, and X represents any of the 19 L-amino acids (cysteine was omitted). Accordingly, the library was organized as an array of 80 mixtures, each one containing 6859 peptides. The total chemical diversity of the library was 137 180 peptides.

Peptide mixtures of the α-helix-restricted library prevent the assembly of the SNARE complex

Peptide mixtures were screened for their ability to prevent the formation or alter the stability of the *in vitro*-reconstituted SNARE core complex. The ternary complex was assembled *in vitro* by incubating the recombinant SNARE proteins at a molar ratio of 1:1:0.1 (VAMP/syntaxin/SNAP25) at 4 °C for 1 h. Complex-formation was analysed by SDS/PAGE as the appearance of a protein band of 75 kDa that was resistant to SDS-mediated denaturation, but sensitive to temperatures of ≥ 90 °C (Figure 1B). The three SNARE proteins assembled into a ternary complex in both the absence and the presence of peptide mixtures, although the amount of SNARE complex assembled was lower in the presence of 2 mg/ml of some peptide mixtures (Figure 1B). Quantification of these results for the entire library is depicted in Figure 2.

When used in concert, the data derived from the screening suggested the chemical identity of the bioactive peptides in the library [24,34,35]. As shown, several peptide mixtures inhibited the formation of the SNARE complex by ≥ 50 % at 2 mg/ml. Because the number of plausible active peptides was too large (5 × 8 × 6 × 8 = 1920), we assayed the active mixtures at 1 and 0.5 mg/ml to select the most potent amino acid sequences (Figure 3). We found that Glu (E) and Phe (F) residues were preferred at position 6 of the peptide scaffold, Leu (L) and Gln (Q) at position 9, Tyr (Y) at position 10, and Trp (W) and Phe (F) at position 14. Taken together, these results suggest

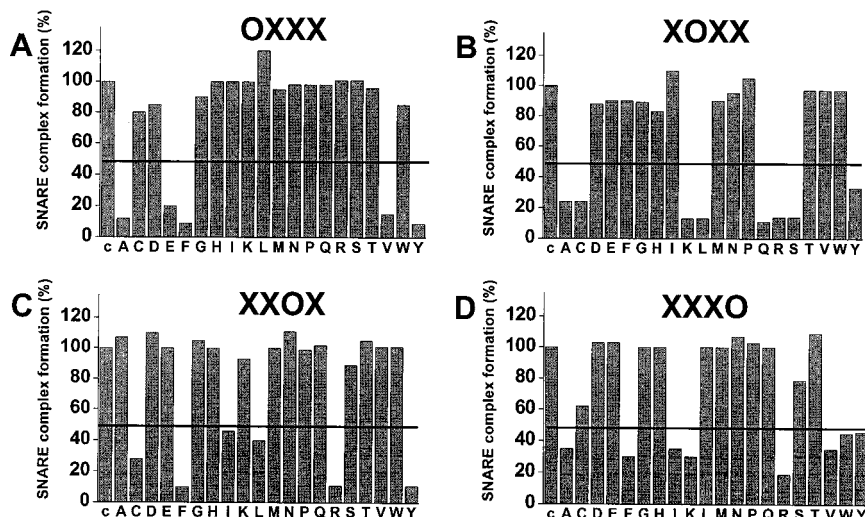


Figure 2 Screening of the combinatorial library identifies peptide mixtures that prevent the formation of the SNARE core complex

The percentage SNARE complex formation is represented as a function of the amino acid used in the defined position of the library (O). The indicated residues denote positions 6, 9, 10 and 14 of the peptide scaffold (see Figures 1 and 4). Ternary complex formation was evaluated by SDS/PAGE as the amount of the 75 kDa protein band. Gels were air-dried and scanned for quantification. Data were normalized to the signal exhibited by control samples (c), which denotes the formation of the protein complex in the absence of peptide mixtures. Peptide mixtures were assayed at 2 mg/ml. Horizontal lines represent the arbitrary cut-off limit ($\geq 50\%$) to consider a mixture as active.

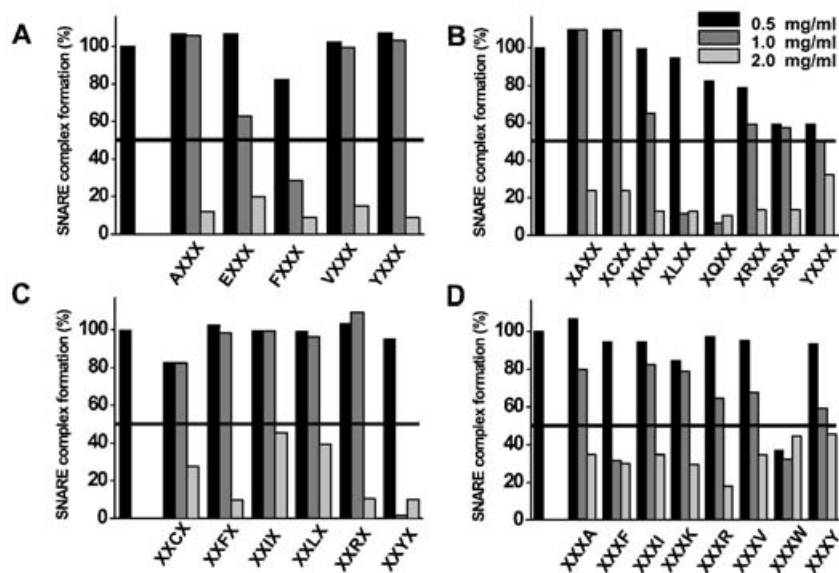


Figure 3 Dose-dependence relationship of inhibitory activity of active mixtures in the library

For library deconvolution, the blockade activity of peptide mixtures was determined at 2, 1 and 0.5 mg/ml. SNARE complex formation is represented as a function of the amino acid at the defined position. The ternary complex was defined as the band of 75 kDa. Horizontal lines represent the arbitrary cut-off of inhibitory activity (as in Figure 2). See Figures 1 and 4 for further details.

a family of eight amino acid sequences (Figure 4A), resulting from all possible combinations of the active residues identified in the deconvolution process, as inhibitors of SNARE complex formation. These peptides are referred to as the amino acids selected for each combinatorialized position of the sequence [$O^6O^9O^{10}O^{14}$] (Figure 4A).

The identified α -helical peptides are potent inhibitors of SNARE complex formation

We next synthesized all 17-mer peptides identified by the screening and determined whether they blocked the assembly

of the *in vitro*-reconstituted SNARE core complex. As illustrated in Figure 4(B), all amino acid sequences affected the formation of the core complex to different extents in a concentration-dependent manner. The most potent sequence was Ac-SAAE-AFAKLYAEAFKAG-NH₂ (referred to as [$F^6L^9Y^{10}F^{14}$]), which, at 0.5 mg/ml, fully blocked ($95 \pm 6\%$) the formation of the SNARE complex (Figures 4B and 4C). All other sequences exhibited significantly lower ($\leq 50\%$) inhibitory activity (Figure 4C).

We next determined, by CD, the content of α -helical structure in peptides [$F^6L^9Y^{10}F^{14}$] and [$E^6Q^9Y^{10}W^{14}$] (Figure 5). As illustrated in Figures 5(A) and 5(B), both peptides show a maximum at 190 nm and two minima at 210 and 222 nm, characteristic of

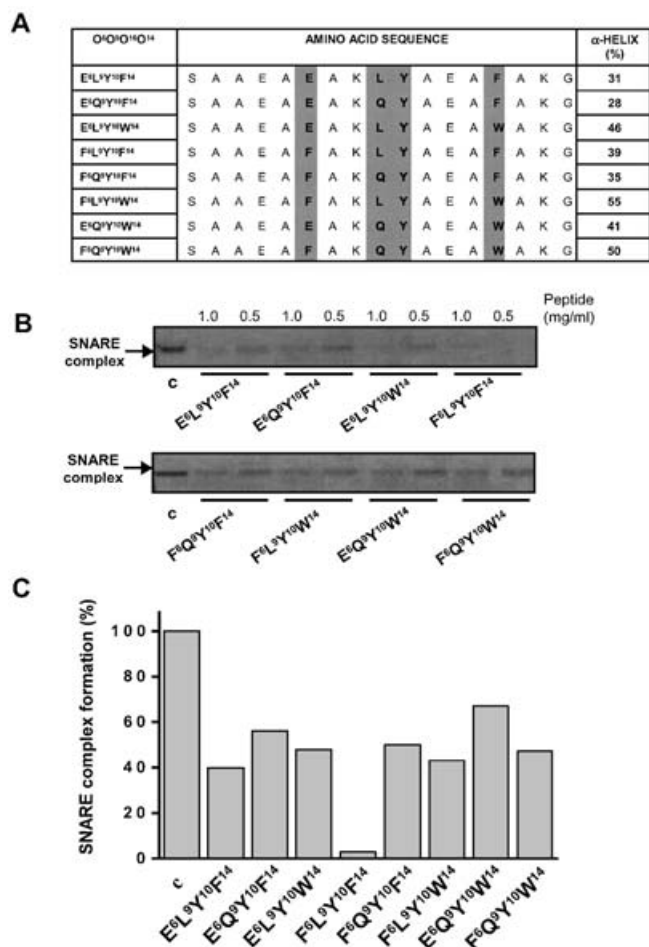


Figure 4 Identified 17-mer peptides are inhibitors of SNARE core complex formation *in vitro*

(A) Amino acid sequences of the peptides identified from the screening of the library. $[O^0Q^9O^{10}Q^{14}]$ represent the amino acids selected at the combinatorialized positions of the sequence. α -Helix (%) denotes the percentage of α -helical secondary structure estimated by the AGADIR program. (B) Inhibition by the identified peptides of SNARE core complex assembly *in vitro*. Peptides were tested at two concentrations: 1 and 0.5 mg/ml. (C) Quantification of the inhibition of SNARE complex formation by individual peptides at 0.5 mg/ml. Control (c) denotes ternary complex assembly in the absence of the peptides. Dried gels were scanned for quantitative analysis.

α -helical peptides. The intensity and location of these CD signals increased as a function of the concentration of trifluoroethanol, a stabilizer of secondary structure. Quantitative analysis show that both peptides exhibited 30% α -helical content in buffer, which increased to 80% as the percentage of trifluoroethanol was increased (Figure 5C). The propensity to adopt an α -helical structure was in accordance with that predicted by the AGADIR program ($\geq 40\%$) for all active peptides in aqueous solution (Figure 4A). Taken together, these results indicate that the identified α -helical peptides are inhibitors of SNARE core complex assembly, and suggest that they may modulate neuronal exocytosis.

The $[F^6L^9Y^{10}F^{14}]$ peptide inhibits neuronal exocytosis

To evaluate the *in vivo* activity of the identified peptides, we studied the inhibitory potency of the most active peptide,

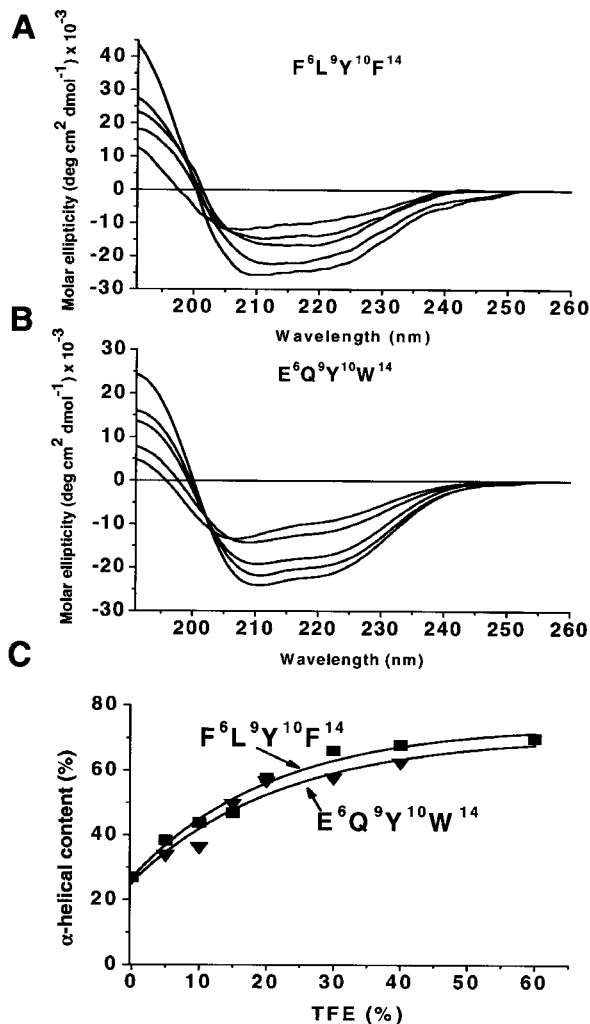


Figure 5 Active peptides have an α -helical structure

(A, B) Far-UV CD spectra of peptides $[F^6L^9Y^{10}F^{14}]$ and $[E^6Q^9Y^{10}W^{14}]$ at increasing percentages of trifluoroethanol (0, 5, 10, 20 and 40%). The peptide concentrations were $5 \mu\text{M}$ $[F^6L^9Y^{10}F^{14}]$ and $20 \mu\text{M}$ $[E^6Q^9Y^{10}W^{14}]$ in 20 mM Tris, pH 8.0, and 10% (v/v) acetonitrile. CD spectra represent the average of five scans, and were corrected for the contribution of the buffer. (C) Quantification of the α -helical content as a function of the percentage of trifluoroethanol (TFE). α -Helical values were obtained as described previously [23].

$[F^6L^9Y^{10}F^{14}]$, and of the lower-activity counterpart $[E^6Q^9Y^{10}W^{14}]$ on the Ca^{2+} -dependent release of catecholamines from detergent-permeabilized chromaffin cells. Incubation of digitonin-treated cells with either peptide resulted in the dose-dependent inhibition of both adrenaline and noradrenaline exocytosis (Figure 6A). Peptide $[F^6L^9Y^{10}F^{14}]$ was more potent at abrogating regulated catecholamine release than peptide $[E^6Q^9Y^{10}W^{14}]$, consistent with the higher potency of the $[F^6L^9Y^{10}F^{14}]$ peptide in inhibiting SNARE complex formation. To further underscore this finding, we investigated whether both peptides block the Ca^{2+} -evoked release of L - $[^3\text{H}]$ glutamate from intact hippocampal primary cultures. As illustrated in Figure 6(B), the K^+ -induced depolarization of hippocampal cultures in the presence of 2.0 mM extracellular Ca^{2+} resulted in the release of L - $[^3\text{H}]$ glutamate. Incubation of neuronal cultures with $10 \mu\text{M}$ $[E^6Q^9Y^{10}W^{14}]$ peptide for 60 min did not affect the K^+ -evoked secretion of L - $[^3\text{H}]$ glutamate. In marked contrast, addition of the $[F^6L^9Y^{10}F^{14}]$ peptide to the neuronal cultures blocked L - $[^3\text{H}]$ glutamate

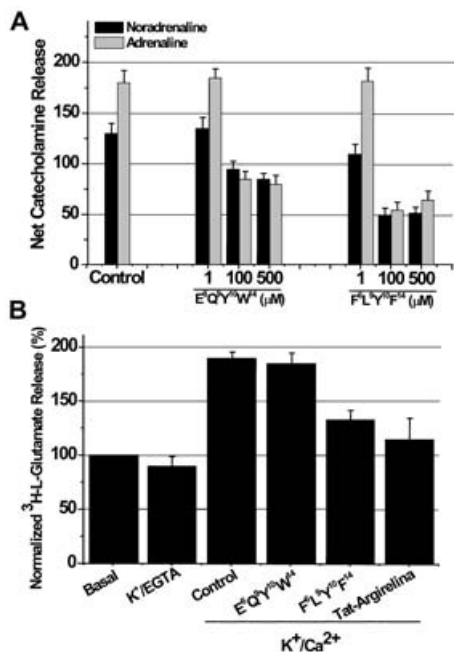


Figure 6 The active peptides inhibit regulated exocytosis

(A) Blockade of catecholamine release from detergent-permeabilized chromaffin cells by peptides [F⁶L⁹Y¹⁰F¹⁴] and [E⁶Q⁹Y¹⁰W¹⁴]. Chromaffin cells were permeabilized with 20 μM digitonin for 5 min. Peptides were administered during detergent permeabilization at the indicated concentrations. Catecholamine release was evoked by 10 μM Ca²⁺. Basal release was measured in the presence of 5 mM EGTA. Adrenaline and noradrenaline were separated by HPLC and quantified with an electrochemical detector. Data are given as means ± S.E.M. for n ≥ 3. (B) Inhibition of L-glutamate release from intact hippocampal neurons by the most active peptide. Neuronal cultures were loaded with L-[³H]glutamate for 90 min, and incubated with 10 μM peptide for 60 min in BSS at 37 °C. L-[³H]glutamate release was evoked by K⁺-mediated depolarization (56 mM KCl) in the presence of 2.0 mM extracellular Ca²⁺. Ca²⁺-independent L-[³H]glutamate release was obtained in the presence of 0.5 mM EGTA. Measurements of radioactivity were normalized to those obtained in non-stimulated neurons (Basal). Values are given as means ± S.E.M. (n = 3).

exocytosis by ≥ 50%. Notably, the potency of blocking of neurosecretion by [F⁶L⁹Y¹⁰F¹⁴] was similar to that exerted by peptide YGRKKRRQRRRQGAGGEEMQRR-NH₂ ('Tat-Argireline'), which contains the SNAP25 sequence EEMQRR-NH₂ fused to the Tat translocating sequence from HIV that confers cell permeability. The sequence Ac-EEMQRR-NH₂, also known as Argireline, has been shown to block catecholamine release from detergent-permeabilized chromaffin cells [10]. Taken together, these results indicate that the 17-mer peptide [F⁶L⁹Y¹⁰F¹⁴] (Ac-SAAEAFKLYAEAFKAG-NH₂) is a cell-permeable, potent inhibitor of neuronal exocytosis.

DISCUSSION

A combinatorial strategy has been used to identify short sequences that target the SNARE complex, thus acting as down-regulators of neuronal exocytosis. For this task, a 17-mer, mixture-based, α-helix-restricted peptide library comprising 137 180 sequences was designed and screened. The choice of maintaining an α-helical conformation was due to the well-known four-helix bundle structure that comprises the SNARE core complex [4,5]. Hence peptides with a propensity to fold into an α-helical secondary structure appear to be suitable candidates to modulate

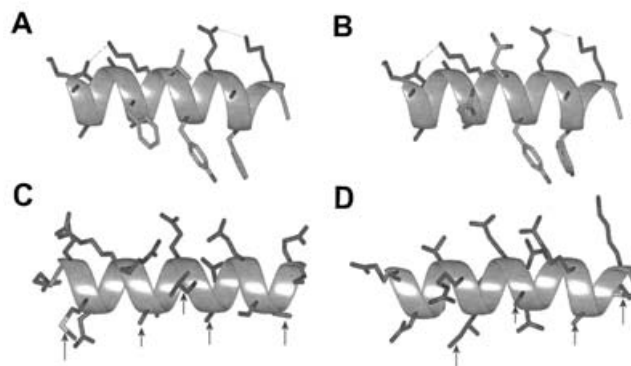


Figure 7 α-Helical models

Models are shown for the most (SAAEAFKLYAEAFKAG; A) and least SAAEAEAKQYAEAWKAG; B) active peptides for inhibition of SNARE complex formation, and for peptides patterned after the N-terminus (EEMQRRADQLADESLES; C) and C-terminus (DSNKTRIDEANQRATKM; D) of SNAP25. The helical structure for SNAP25 peptides was obtained from the three-dimensional structure of SNAP25 in the SNARE complex. Arrows indicate hydrophobic residues involved in core coiled-coil interactions in the SNARE complex.

the stability and/or formation of the ternary complex. A salient contribution of the present study is the identification of α-helical peptides that efficiently prevent the *in vitro* assembly of the SNARE core complex. Notwithstanding, the most potent 17-mer (Ac-SAAEAFKLYAEAFKAG-NH₂) blocked Ca²⁺-triggered exocytosis in detergent-permeabilized chromaffin cells and in intact primary cultures of hippocampal neurons. The inhibitory activity of this peptide was virtually identical to that exhibited by peptides patterned after protein domains of SNARE proteins [3,8–19]. It should be noted that these newly identified sequences do not show sequence similarity to any of the primary structures of the SNARE proteins. This conclusion is relevant, since thus far only peptides mimicking specific domains of SNARE proteins have been shown to be potent modulators of SNARE complex assembly and, in turn, of neurosecretion.

Cumulative evidence has made a case for α-helical and coiled-coil secondary structure propensities as determinants of SNARE-complex interactions. Indeed, active peptides acting on the ternary complex that abrogate exocytosis fulfil these key properties [20]. The 17-mer sequences identified from the combinatorial screen exhibit a significant propensity to fold into an α-helix, as predicted by the well established AGADIR program [33] and confirmed by CD analysis of two representative peptides. A computer model of these peptides illustrates that the structured helices have a hydrophobic face which may participate in coiled-coil interactions (Figures 7A and 7B), similar to those exhibited by peptides patterned after the N- and C-terminal ends of SNAP25 (Figures 7C and 7D). Thus the identified peptides also appear to have the two properties described for SNARE core complex assembly [36,37]. A homology BLAST search in non-redundant protein databases did not identify any sequence with similarity to that exhibited by the α-helical peptides. Thus these peptides provide novel scaffolds that can modulate the protein-protein interactions involved in vesicle fusion.

An important property of peptides that abolish neuronal exocytosis by targeting the SNARE core complex is the sequence specificity of their activity. Randomly generated sequences with the same amino acid composition as active peptides do not inhibit ternary complex formation or neurosecretion [9,15,16]. Our results are also consistent with this tenet, as indicated by the amino acid requirements of the combinatorialized positions for

efficient blockade activity. As reported, the most active sequence was [F⁶L⁹Y¹⁰F¹⁴]. Replacement of Phe-6 by Lys, of Lys-9 by Gln or of Phe-14 by Trp notably disrupted the inhibitory activity of the peptide *in vitro* and *in vivo*. A plausible explanation for these observations is that incorporation of a polar or charged residue into the hydrophobic face of the α -helix may disrupt its stability. Nonetheless, the propensity to fold into an α -helical conformation was similar for all sequences, as determined by AGADIR (30–50%) and confirmed by CD for the most and least active peptides. Hence an alternative explanation for the distinct potencies could invoke the interaction of the peptide with its target, i.e. the SNARE complex. Future studies will address this important question and attempt to identify the structural determinants involved in the molecular recognition of both entities.

It is significant that the most active peptide inhibited the Ca²⁺-evoked release of L-glutamate from intact hippocampal neurons, thus indicating that the peptide is cell-permeable and stable in the cytosol. Blockade of regulated exocytosis was observed at a concentration as low as 10 μ M, which is 10-fold lower than that required to target the SNARE complex *in vitro*. This finding is consistent with the notion that the *in vivo* exocytosis-competent SNARE complex is in the *trans* configuration, while *in vitro* the *cis* form predominates. It has been reported that the *trans*-SNARE complex is energetically less stable than the *cis* form, and thus more amenable to modulation by small molecules [1,2,3,19,36].

Conformation-constrained libraries have been used to search for structured peptides [24,36], and to identify peptides with endotoxin-neutralizing activity, antibiotics and enhancers of viral infectivity [21–23,38], thus substantiating the notable potential of these libraries as sources of active structured compounds. Our findings further support this tenet, and provide the rapid identification of cell-permeable small peptides that modulate neurosecretion by targeting the essential SNARE core complex. These peptides may be useful pharmacological tools with which to study synaptic transmission and, in turn, may be used as scaffolds to develop drugs that attenuate neuronal hyperactivity, such as that occurring in spasmodic disorders [7].

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