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A family of cell-adhering peptides homologous to fibrinogen C-termini

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ABSTRACT

A family of cell-adhesive peptides homologous to sequences on different chains of fibrinogen was investigated. These homologous peptides, termed Haptides, include the peptides C β , preC γ , and C α E, corresponding to sequences on the C-termini of fibrinogen chains β , γ , and α E, respectively. Haptides do not affect cell survival and rate of proliferation of the normal cell types tested. The use of new sensitive assays of cell adhesion clearly demonstrated the ability of Haptides, bound to inert matrices, to mediate attachment of different matrix-dependent cell types including normal fibroblasts, endothelial, and smooth muscle cells. Here we present new active Haptides bearing homologous sequences derived from the C-termini of other proteins, such as angiopoietin 1&2, tenascins C&X, and microfibril-associated glycoprotein-4. The cell adhesion properties of all the Haptides were found to be associated mainly with their 11 N-terminal residues. Mutated preC γ peptides revealed that positively charged residues account for their attachment effect. These results suggest a mechanism of direct electrostatic interaction of Haptides with the cell membrane. The extended Haptides family may be applied in modulating adhesion of cells to scaffolds for tissue regeneration and for enhancement of nanoparticle transfection into cells.

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1. Introduction

The interaction with biological matrices is vital for cell activity and survival [1–3]. Cells adherence to extra-cellular matrix is mediated mostly through interaction with cell membrane receptors termed integrins which bind to specific sequences such as RGD motif [2].

Fibrin(ogen) generates cell adhesion to different matrix-dependent cell types, including fibroblasts (HF), endothelial cells (EC), and smooth muscle cells (SMC) [4–7]. Though fibrinogen was claimed to attach cells by integrins, recombinant fibrinogen lacking RGD sequences still mediate cell adhesion [8]. This led us to look for other cell attachment epitopes. We found highly preserved sequences at the C-termini of the β , γ , and α E fibrinogen chains. The peptides, based on these sequences C β (β 441–461), preC γ (γ 373–392), and C α E (α E 847–866) were termed “Haptides”. Their cell attachment response is not mediated by integrins (Table 1) [9].

Soluble Haptides exhibit cell permeation and nano-particles internalization properties. This does not affect cells survival and proliferation rate. When bound to matrix the Haptides attach matrix-

dependent cells [9] which improves implants integration [10]. They may also accelerate fibrin polymerization and clot formation [11].

So far most cell adhesion assays have been based on monitoring plating efficiency of trypsinized cells to surfaces coated with the ligand adsorbed and not bound to the tested surface [12–15]. Moreover, different cell membrane surface determinants are compromised. We used a quantitative cell adhesion assay. Sepharose beads (SB) chemically coated with predetermined amounts of the tested ligand are placed over the intact cell layer. The proportion of coated SB immobilized to the cell layer, through the cell-adhesive ligand, could be evaluated over time to reflect its cell binding properties [9,16] (Fig. 1).

In the current study we extend the Haptides family to include additional homologous sequences derived from the C-termini of other proteins: microfibril-associated protein-4 (MFA), angiopoietins I&II and different isotypes of tenascins [13,17]. Testing mutated Haptides and observing conformational changes in membrane-like environment we concluded that the Haptides mechanism of action involves a direct interaction with the cell membrane. This interaction is mediated mostly by electrostatic forces.

2. Materials and methods

2.1. Chemicals and reagents

Tissue culture media, serum, bovine serum albumin (BSA) were purchased from Biological Industries (Beit-HaEmek, Israel), Sigma

Abbreviations: Fibrin(ogen), fibrin and/or fibrinogen; SB, sepharose beads; FITC, fluorescein isothiocyanate; FL, fluorescein; HF, normal human fibroblasts; BAEC, normal bovine aortic endothelial cells; SMC, smooth muscle cells; DPC, dodecylphosphocholine micels; FCS, fetal-calf-serums.

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Table 1

Haptidic sequences derived from the C-termini of fibrinogen and other proteins.

Name	Sequence	Net positive charge	Hydrophobicity (% hydrophobic residues)	Protein
Highly cell adhesive				
preC γ	KTRVWYSMKTTMKIIPFNRL	+6	0.35	Fibrinogen
C β	KGSWYSMRKMSMKIRPFPPQ	+5	0.33	Fibrinogen
Cmfa	KGFYYSLKRTMKIRRA	+5	0.35	MFA
Mean		+5.33	0.34	
Medium cell adhesive				
C α E	RGADYSLRAVRMKIRPLVTQ	+4	0.45	Fibrinogen
CtenC	KGHEHSIQFAEMKLRPSNFR	+3	0.35	Tenascin C
CtenX	KGFESVPTTEMKLRPNFR	+3	0.4	Ten-x
Cang1	KGPSYSLRSTTMMIRPLDF	+2	0.37	Angiopoietin 1
Cang2	KSGSYSLKATTMMIRPADF	+2	0.47	Angiopoietin 2
Mean		+2.8	0.41	
Control				
C α	SEADHECTHSTKRGHAKSRP	2.5	0.2	Fibrinogen

Positively charged residues are labeled in blue. Negatively charged residues are labeled in red. Hydrophobic residues are labeled in brown. Polar residues are labeled in black and aromatic residues in green. The control non-Haptide peptide homologous to a sequence the C terminal of the α chain (C α) is also presented.

Chemicals (Israel and St. Louis, MO) and GIBCO (Grand Island, New York, NY).

2.2. Cell cultures

The cell types used in the present study were cultured as previously described [4,9]. RPMI 1640 and DMEM media were used, typically +10% fetal-calf-serums (FCS) with high or low glucose, as dictated by the experiments and cell types tested. Standard additives were added to media, including Penicillin–Streptomycin with Nystatin, L-glutamine, MEM–Eagle vitamin solution and non-essential amino-acids (Beit-HaEmek). Normal HF (supplied by the department of genetics at Hadassah Hospital) were isolated from skin biopsies of young normal volunteers and cultured for no more than seven passages in high-glucose DMEM + additives. SMC and BAEC were isolated from fresh thoracic aortas collected at slaughterhouse from sacrificed young animals and were kept in culture for up to 12–15 passages in low-glucose DMEM + additives.

2.3. Peptide synthesis

Peptides were synthesized using a ABI 433A peptide synthesizer, as previously described [18], or using a Liberty MAPS (Microwave Assisted Peptide Synthesizer, CEM) as previously described [19]. Some of the peptides, such as the fibrin related Haptides and Cmfa, were also purchased from SynPep Corporation (Dublin, CA). All peptides were >90% pure as determined by HPLC and MALDI-TOF mass-spectrometry.

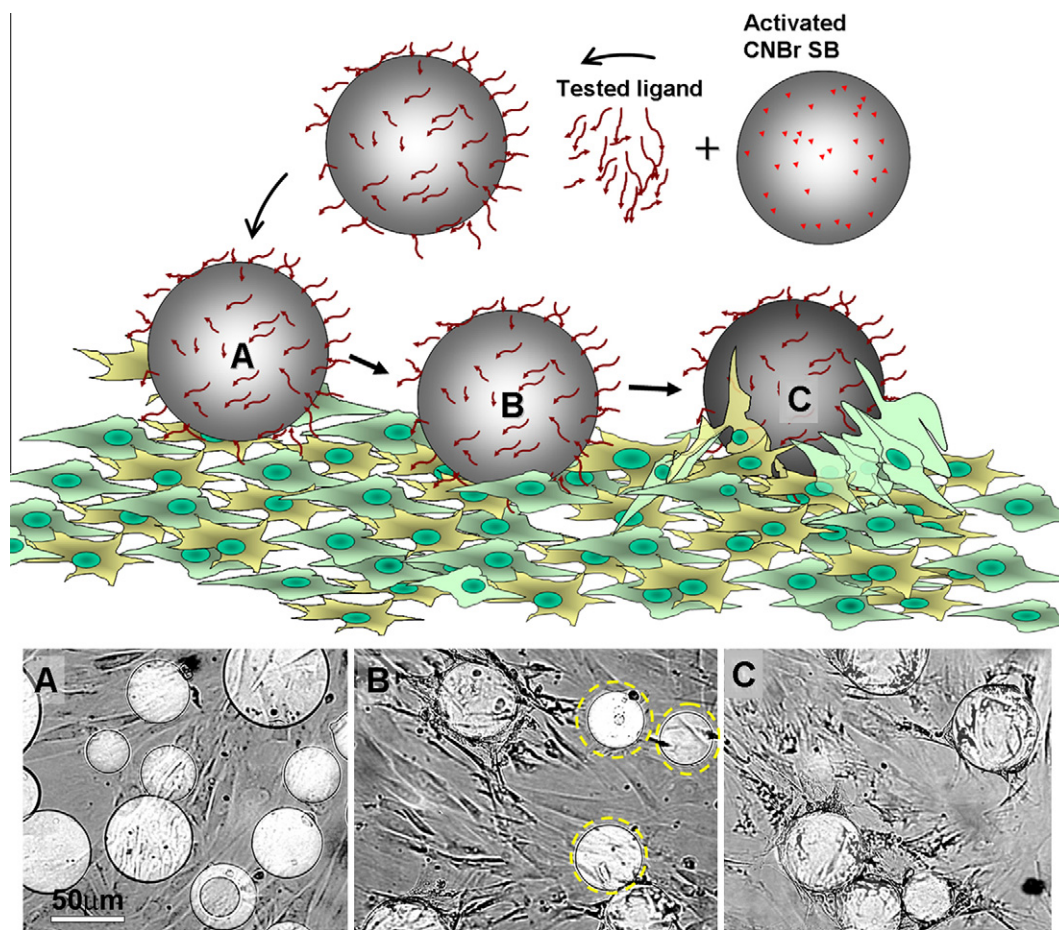


Fig. 1. (A) Schematic description of SB-ligand cell attachment assay: upper panel is a schematic representation of the assay and lower part shows relevant light micrographs with reference to the upper panel, as follows. (A) Initially the transparent SB float freely over the intact cell layer. (B) The SB coated with active ligand initiate attachment to the monolayer cells within 0.5–2 h. At this stage, some SB with less potent ligands may still float freely (circled broken line). (C) Most of the SB coated with highly potent cell binding ligands are immobilized to the cultures within 4 h and completely attached to the cell layer within 24 h. With time the cells physically respond to the coated SB, mount on them and further anchor them tightly to the cell monolayer.

Table 2
Homologous short 11mer Haptidic sequences derived from the C-termini of fibrinogen and other proteins.^a

Name	Sequence	Charge	Hydrophobicity (% hydrophobic residues)	Protein
High cell attachment (short)				
preC _{γ11}	KTRVWYMKTT	+4	0.18	Fibrinogen
Cβ ₁₁	KGSWYSMRKMS	+3	0.36	Fibrinogen
Cmfa ₁₁	KGFYYSLKRT	+2	0.27	mfa
Mean		+3	0.27	
Low cell attachment (short)				
Cang1 ₁₁	KGPSYSLRSTT	+2	0.18	* Angiopoietin 1
No cell attachment (short)				
CtenX ₁₁	KGFESVPFTE	−1	0.45	Ten-x
Cβ ₁₁₋₂₀	SMKIRPFPQQ	+2	0.27	Fibrinogen

^a See legend for Table 1.

2.4. Coupling of ligands to activated CNBr-activated SB

Haptides or proteins were covalently bound to CNBr-activated Sepharose 4B beads (Pharmacia, Piscataway, NJ) as previously

described [4,9]. Unless otherwise indicated, 6 mg peptides were bound to 1 ml activated SB.

2.5. Coupling Haptide to bovine serum albumin covalently attached to SB (SB-alb)

EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) chemistry was used to bind Haptides to albumin covalently bound to SB. Fifty μL of SB-albumin were incubated with 50 μL of 100 μg/g FITC labeled preC_γ (^{FITC}preC_γ). EDC powder (2 mg) was then added. Following 30 min of incubation in RT, the SB were washed twice with PBS.

2.6. Light and fluorescence microscopy

Light and fluorescence images were obtained with a DS-R1 color camera with DS-L2 controller mounted on Eclipse TE200 microscope with Nomarsky optics + fluorescence set-up (all from Nikon, Japan).

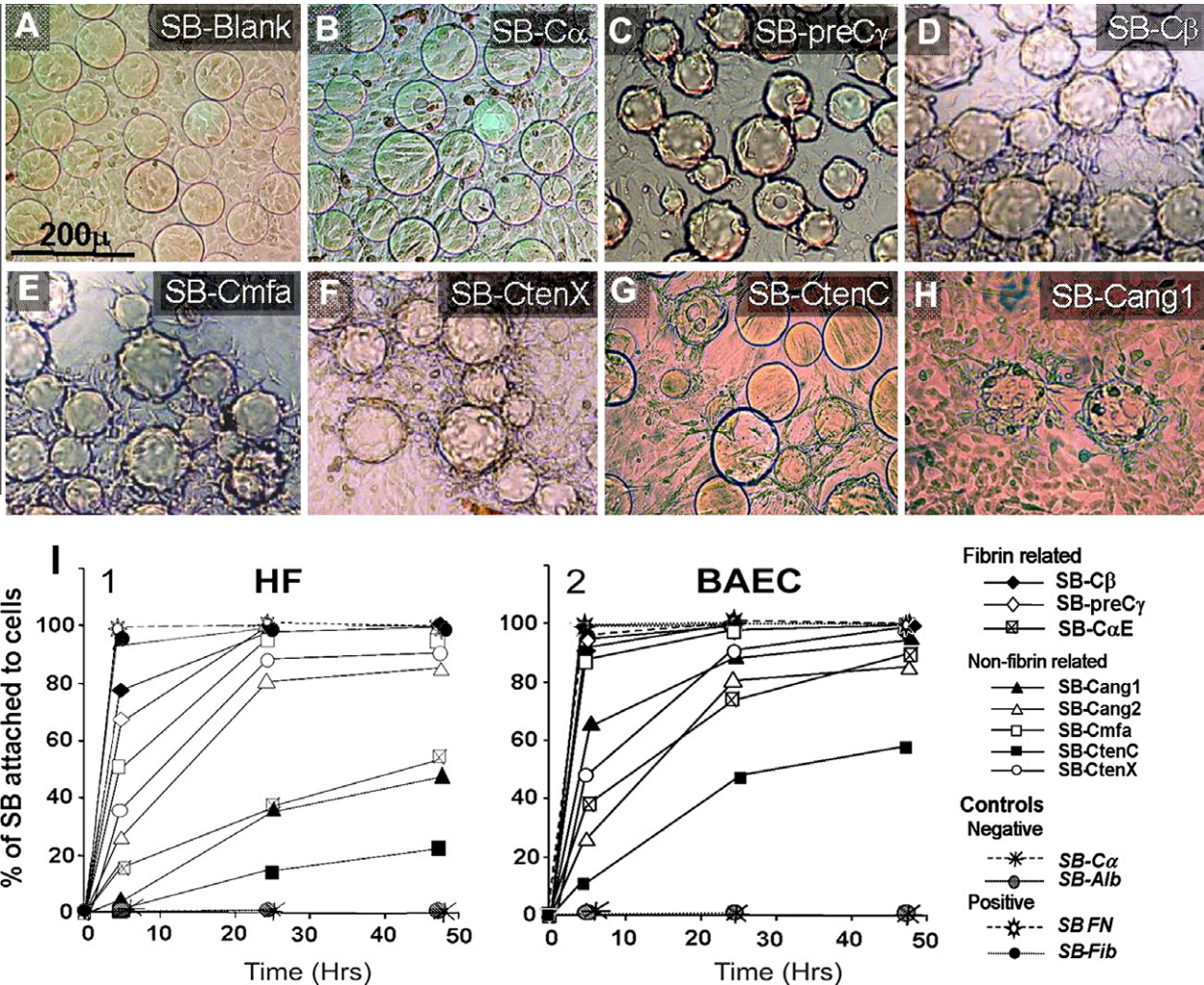


Fig. 2. The extended family of Haptides. (A–H) Nomarsky-optics microscopy of SB covalently coated with ligands, placed on cultured BAEC after 72 h of incubation. SB-albumin (blank) or SB-Cα float freely over the cell layer. SB coated with the active Haptides preC_γ, Cβ, Cmfa, CtenX, CtenC, and Cang1, are immobilized to the BAEC (I) summary of attachment-response kinetics of HF cells (I-1) and BAEC (I-2) by the SB-ligand attachment assay. Both the early response (marked by broken line) and the increased adhesion with time are presented. SB coated with fibronectin (FN) and fibrinogen (Fib) served as positive control. SB-Cα or with albumin (Alb) served as negative controls and showed no cell attachment response. Cβ, preC_γ, and Cmfa exhibit the highest attachment kinetics similar to SB-Fib and SB-FN. Cang1 and Cang2 and CtenX exhibited lower cell attachment activity. The relatively lowest cell attachment response was observed with CtenC. The relative standard error of this assay is expected to be within 6% and is the outcome of the number of beads counted in each well in triplicates.

2.7. Assay of cell attachment to tested ligands on SB

Cell attachment activity of the tested ligands was assayed with the sepharose beads (SB) technique [4,9,16]. The basis of the method is demonstrated in Fig. 1. A ~200–300 beads coated with the tested ligand were dispersed on a near confluent cell monolayer in 12 well plates by gentle shaking for 1 min. At different time points, from 2 h onward the number of SB tethered to cell layer was counted with an inverted phase microscopy and the ratio of the number of SB attached to the cell layer relative to the total number of SB in each well was calculated. Only SB coated with a potent cell attachment ligand were attached to the cell layer, so that the background of the assay and negative control was virtually zero. This assay enabled to observe both short and longer time cell attachment responses, which further stress the differences between the less-active and the highly active peptides. From 24 h onward the SB coated with potent ligands became engulfed by the cells while the non-responders uncoated SB, or SB coated with non-active ligands, rolled on the cell culture without interacting with the cells (Fig. 1). The % of the count of SB attached to the cell layer at different time intervals provided a quantitative estimate of the kinetics of the cell attachment responses. At least three wells were measured for each variant and each experiment was repeated at least three times.

2.8. Assay of cell attachment to different Ala-substituted preC γ peptides in suspension culture

Samples of 35 μ l SB-peptides (6 mg/ml) were prepared in 15 ml tubes, washed with PBS \times 3 and then with full medium. Trypsinized cells (2×10^5) were added to all the tubes in triplicates and incubated in rotation for 2 h. Then the SB were allowed to settle and the medium was removed carefully and rinsed once with medium. Fresh medium was then added and the cells were further incubated for 24 h. Cell number attached to the SB was then determined with the modified procedure of MTS assay (Promega cell proliferation Kit, Promega WI, USA) as previously described [16], using a previously prepared calibration curve for the relevant cells.

2.9. Circular dichroism (CD) studies

Peptides were dissolved in 10 mM phosphate buffer, pH 7.2, 32.5 mM MgSO₄. Peptides concentrations were 65 μ M for preC γ , 63 μ M for C β , and 76 μ M for Cmfa. The concentrations of the preC γ mutated peptides were 70 μ M of R3AK8AK9A and W4AY5A, 66 μ M of R3A, K8A, and K9A, 68 μ M of W4A, Y5A, and K8AK9A. Concentrations were determined using a UV spectrophotometer (Shimadzu, Kyoto, Japan). CD spectra were recorded as previously described [19]. The secondary structure of the Haptides (Table 3) with and without DPC was calculated using DichroWeb [20] with the SELCON3 analysis algorithm.

2.10. Gene-bank database

The databases for fibrinogen sequences in human and other species with the use of the BLAST-P protein sequences were searched with the use of Gapped BLAST and PSI-BLAST [21] and 3D models were built with the help of MMDB and Cn3D, a helper application for three-dimensional structures from NCBI's Entrez retrieval service [22].

3. Results

3.1. Search for new homologs of Haptides sequences

A search in the NCBI gene bank revealed homologous sequences to fibrinogen related Haptides in the C-termini of various

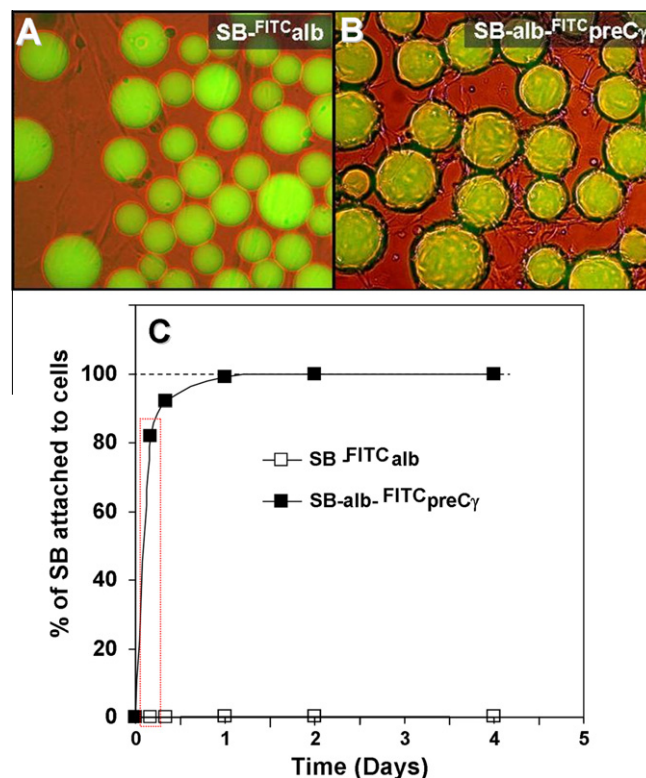


Fig. 3. Cell attachment to albumin coated with Haptides. Nomarsky dim light combined with fluorescence micrographs of the response of monolayer BAEC to SB coated with: (A) FITC labeled bovine serum albumin. (A) SB-FITC Alb exhibit no adhesion response. (B) The SB-Alb-FITC preC γ got firmly attached to the monolayer cells through their coating with FITC preC γ ; (C) the binding kinetics of SB-Alb-FITC preC γ , in comparison to the cell binding of SB-FITC Alb. The albumin coated SB and the FITC did not show any cell binding properties while the binding of the preC γ to the albumin on the SB rendered the coated SB high cell attachment properties.

non-related proteins. Those include angiopoietin 1&2, tenascins C&X, and microfibril-related protein-4. We synthesized the peptides derived from these sequences and termed them Cang1, Cang2, CtenC, CtenX, and Cmfa, respectively (Table 1).

3.2. Haptides in other proteins mediate cell adhesion similar to fibrinogen-derived Haptides

The rate that the intact cell layer immobilizes the ligand coated SB can follow up, accurately, the attachment kinetics of the SB-li-

Table 3

Secondary structure content of the leading Haptides and preC γ mutated peptides.

Name	%Helix	%Strand	%Turn	%Disordered
Cmfa	68.9	1.7	10.8	18.6
preC γ	78.4	0.6	6.4	14.7
preCγ substituted peptides				
K8A	79.0	0.2	6.3	14.5
K9A	77.9	1.0	6.2	14.9
R3A	34.2	11.3	23.4	31.2
K8AK9A	80.7	−0.5	5.9	13.9
R3AK8AK9A	80.2	−0.2	5.6	14.4
W4A	20.7	23.9	19.3	36.1
Y5A	80.2	−0.1	5.5	14.4
W4AY5A	20.8	24.0	19.0	36.2

The secondary structure content of preC γ , Cmfa, and the alanine-substituted preC γ peptides in 20 mM DPC was calculated using DichroWeb [20] with the SELCON3 analysis algorithm.

gand to relevant cells. SB coated with the new Haptides Cmfa, Cang1, CtenX, and CtenC, demonstrated cell attachment responses similar to those seen with the fibrinogen related Haptides C β and preC γ . SB bound to negative controls such as albumin, or a non-related C α peptide derived from fibrinogen α C-terminus or blank SB, had no interactions with the cells (Fig. 2A–H). Assay of the immediate attachment of SB-ligand to the monolayer provides the basic information on their binding properties. The ability to examine this parameter at different time points provides further information on the kinetic profile of cell attachment to the tested ligands (Fig. 2I–L&2). Fibronectin and fibrinogen monomers bound to SB (SB-FN and SB-Fib, respectively) served as positive controls.

Within the first 5 h the new Haptide Cmfa exhibited the highest cell attachment potency (87%) with BAEC cells, similar to the most active fibrinogen Haptides C β (90%) and preC γ (95%). After \sim 50 h those Haptides reached $>90\%$ attachment. Cell attachment of SB-Cang1, SB-Cang2, and SB-CtenX was slower (65%, 25%, and 45%, respectively). Attachment of SB-ligand to HF was also tested. The kinetic of attachment of SB-Cang2 and SB-CtenX was similar to the profile of activity with BEAC. However the response to SB-Cang1 was much lower and even after 50 h only $\sim 40\%$ of the SB were bound to the cells. Yet, in both cell types these peptides showed high activity while within the new extended family of Haptides CtenC was found to be the least active.

3.3. Haptides induce cell attachment of non-cell-adhering proteins

The ability to transform albumin, a non-cell adhesive protein, into highly cell binding agent by coupling Haptides to it was

tested. SB were coated with unlabeled or FITC labeled human albumin (SB-Alb or SB-FITC Alb, respectively). FITC labeled Haptide (F^{ITC}preC γ) was then bound to SB-Alb by EDC coupling technique to form SB-alb-F^{ITC}preC γ . SB-alb-F^{ITC}preC γ demonstrated high cell attachment responses, as opposed to SB-FITC Alb (Fig. 3). This shows that coupling Haptides can render proteins cell binding properties and that FITC by itself is not responsible for this effect.

3.4. The short 11 N-terminal residues of the Haptides are crucial for their cell adhesion activity

To identify the essential residues for the Haptide's adhesion activity, shorter derivatives were designed and synthesized. 11–21 C-terminal residues (11–21AA) of C β showed no cell adhesion. However, 1–11AA of the Haptides, termed C β ₁₁, preC γ ₁₁, Cmfa₁₁, CtenC₁₁, and Cang1₁₁, respectively, demonstrated significant attachment responses to both BAEC and HF cells (Table 2, Fig. 4A). Thus, we conclude that the N-terminal 11AA are essential for the Haptides activity. Yet, unlike the full size Haptides, the shorter active sequences were less stable and lost $>40\%$ activity in one week of storage at 4 °C. Thus, we concluded that the full sequences render the Haptides more stable.

3.5. The contribution of positively charged residues to cell adhesion activity

To determine the contribution of the positively charged or aromatic residues to the Haptides activity, we designed and synthesized several alanine-substituted derivatives of preC γ (Table 3).

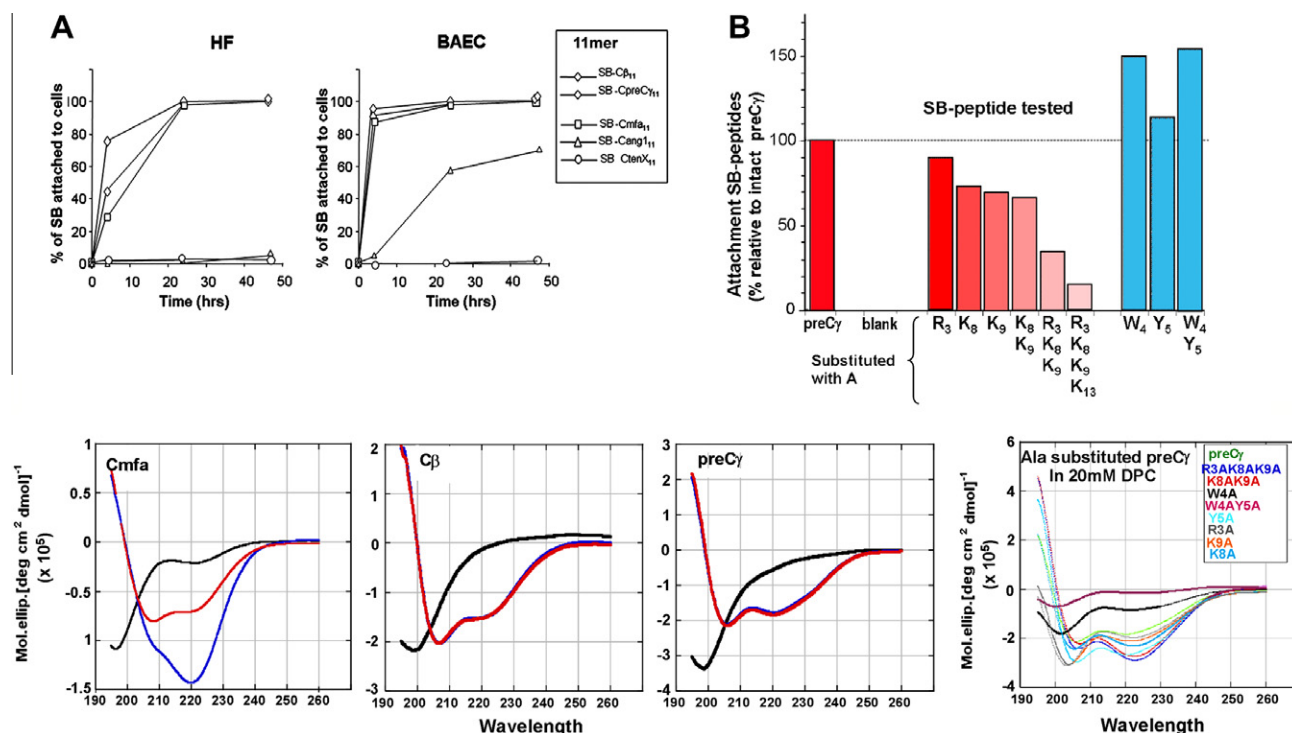


Fig. 4. The main residues contributing to the Haptides activity: (A) attachment-response kinetics of cells to 11mer N-terminal portion of the Haptides (Table 2). The most significant attachment occurred with C β ₁₁, preC γ ₁₁, and Cmfa₁₁. Cang1₁₁ reacted with BAEC but not with HF, and CtenX₁₁ exhibited no cell attachment activity (B) A attachment of bone-marrow derived mesenchymal stem cells to preC γ , and Ala-substituted preC γ . Arg or Lys substitution to Ala resulted in impairment of the adhesion activity, as more positively charged residues were replaced. Replacing aromatic residues at positions 4 and 5 improved the cell attachment response, compared to preC γ , especially in preC γ -W4A preC γ -W4AY5A. (C) Structural analysis of C β , preC γ , and Cmfa in PBS buffer (black), or around 10 mM DPC (blue) or 20 mM DPC (red) by Far-UV circular dichroism. In aqueous conditions of PBS C β , preC γ , and Cmfa exhibited a random coil structure. In the presence of 10 mM DPC or 20 mM DPC. The structure of all peptides tested adopt helical conformation. Increasing the DPC concentration from 10 mM to 20 mM had no significant effect on the structures of preC γ or C β . The spectrum of Cmfa became more helical with the increase of DPC levels. (D) CD measurements of Ala-substituted peptides in 20 mM DPC environment: Lys to Ala-substituted peptides transit from random coil structure to helical conformation. Substituting the aromatic residue Trp at position 4 decreased the helical conformation to 21% both in W4A and in W4AY5A (Table 3).

Mutated preC γ derivatives R3A, K8A, and K9A exhibited 90%, 73%, and 75% activity relative to preC γ , respectively (Fig. 4B). The effect of replacing positively charged residues was additive. Cell attachment mediated by the mutated preC γ derivatives K8AK9A, R3AK8AK9A, and R3AK8AK9AK13A had decreased activity to 68%, 33%, and 15%, respectively, relative to preC γ . The aromatic residues seemed not to contribute to the Haptide's cell binding activity. Replacement these residues at positions 4 and 5 in preC γ derivatives Y5A, W4A, and W4AY5A resulted with 115%, 150%, and 155% cell binding, respectively, over native preC γ (Fig. 4B).

3.6. Conformational changes of Haptides in micellar environment

Possible structure changes that may hint on the mechanism of action of the Haptides preC γ , C β , and Cmfa were examined by circular dichroism (CD). All tested Haptides exhibited random coil structures in PBS solution at RT in pH 6.8. When DPC, a model for a membrane-like environment, was added, a conformational change towards a helical structure in the above Haptides was recorded (Fig. 4C). These findings may suggest a possible mechanism of direct Haptides interaction with the cell membrane.

CD studies were conducted also on the Ala-substituted derivatives of preC γ to examine their effect on the conformational changes in DPC environment. All mutated Haptides showed a random coil structure in PBS solution. In most positively mutated Haptides, the effect of shifting from random coil to helix around DPC remained (Fig. 4D). Yet, the R3A mutation decreased the helical content from 78% to 34%, as calculated by DichroWeb (Table 3). The fact that both W4A and W4AY5A exhibit a decreased helical structure of 21% indicates that the Trp residue was essential for the shift to a helical structure (Fig. 4D).

4. Discussion

Cell adhesion to matrices can be modulated by specific cell receptors [1,3]. Recently discovered Haptides suggests an additional mechanism for cell adhesion, not associated with integrins [9]. The Haptides sequences are not restricted to fibrin. Haptides seems to play a major role in adhesion of matrix-dependent cells. The extended Haptides family includes Cmfa, Cang1, Cang2, CtenC, and CtenX. The shorter versions of the Haptides (C β ₁₁, preC γ ₁₁, Cmfa₁₁, CtenC₁₁, and Cang1₁₁) represent the core sequence contributing to their main activity (Table 2, Fig. 4A). These sequences may be involved in the interactions of the parent proteins with cell membranes (Table 1).

4.1. Haptides mechanism of action

The Haptides were examined for specific features that distinguish their capability to adhere cells. Highly cell-adhering Haptides (~20mer) have aromatic, positively charged and hydrophobic residues (Table 1). Particularly, the more cell-adhering members of the Haptides family also have more positively charged residues (Table 1). The Ala-substituted preC γ mutated peptides showed the same tendency. The more Ala-substituted positive residues the lesser cell attachment response was observed. This indicates that the main property required for adhesive Haptides is a high net positive charge, while their adhesive activity occurs mostly through electrostatic forces. Hydrophobicity is necessary but not sufficient for activity, as indicated by the hydrophobic CtenX₁₁, which was found to be inactive, probably due to its low residual net charge (−1).

PreC γ , C β , and Cmfa structure shifted from random coil to helix in DPC environment (Fig. 4C). Such transition in DPC was

previously shown for membrane-binding peptidic sequences [23]. This implies that Haptides may be able to interact directly with cell membrane. Disturbing this structural transition in preC γ -W4A and preC γ -W4AY5A (Fig. 4D) did not impair and even slightly improved cell attachment. Thus, the adhesive response of Haptides may be accompanied by a conformational change towards helix, but this change does not seem to be necessarily required.

5. Conclusions

Based on our observations, we proposed a model for Haptides cell-adhering mechanism: Haptides seem to interact directly with the cell membrane without involvement of specific receptors, due to an electrostatic attraction between their net positive charges and the negative charged membrane. While interacting with the cell membrane, Haptides serve as anchors that immobilize cells to the matrix. We assume that subsequently, the matrix-dependent cells secrete extra-cellular molecules (ECM) that stabilize the cell adhesion to the matrix. Eventually, only the ECM secreting cells will remain bound.

Our findings set the basis for therapeutic applications of Haptidic sequences. Due to their human source, the extended family of Haptides is not expected to be immunogenic. Therefore, different Haptides may be used as modifiers of implanted materials to improve their cell attachment properties. Further work should focus on the cell penetrating properties of the whole Haptides family for their application for transduction of materials into cells [4,10].

Acknowledgments

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