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Synthetic peptides containing a conserved sequence motif of the Id protein family modulate vascular smooth muscle cell phenotype

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ABSTRACT

Modulation of smooth muscle cells to a proliferating and migrating phenotype with downregulated α-actin expression is observed upon vascular lesion formation. The Id proteins (inhibitors of cell differentiation) play a role in the development of this phenotype. In contrast, synthetic peptides based on a conserved 11-residue Id sequence trigger the switch to a contractile phenotype that shows reduced cell growth and migration, increased expression of α-actin and decreased Id protein levels.

Vascular disorders and diseases like restenosis, vein graft occlusion, and atherosclerosis are characterized by smooth muscle cells (SMCs) with highly proliferative and migrating phenotype.[1,2] The mechanisms underlying the development of such phenotype are poorly understood; however, the observation that proliferation and differentiation of SMCs are deregulated after vascular injury has stimulated the seeking for modulators of these events.

It has been found that some transcription factors from the helix–loop–helix (HLH) family affect the expression of α-actin, a marker of SMC differentiation.[3] In particular, the E proteins, ubiquitous HLH factors with a basic DNA-binding site N-terminal to the HLH motif (bHLH), positively influence α-actin. The opposite is done by the Id proteins, HLH factors that lack a basic DNA-binding site and inhibit the E-protein-mediated activation of DNA transcription. Also, Id protein overexpression leads to significant increase in SMC proliferation.[4] It is known that, upon dimerization with the Id proteins, the E proteins are devoid of the ability to form ternary complexes with tissue-specific bHLH factors, like the myogenin regulating factor MyoD, and DNA, an event that is required for the transcriptional activation of specific genes.[5,6] Therefore, targeting the Id proteins may represent a strategy to control both SMC proliferation and differentiation, with the aim of restoring a healthy contractile phenotype after vascular injury and disorders. Moreover, these proteins are attractive targets for cancer therapy, as they promote tumor angiogenesis and metastasis.[7,8] Indeed, Id protein inactivation by delivery of an antisense oligonucleotide[9] or by expression of an engineered HLH dimerization partner[10] has been shown to inhibit tumor growth, metastasis, and angiogenesis.

In this work we present peptide-based compounds that interact with the synthetic Id HLH dimerization domain and modulate multiple events in the synthetic phenotype of human SMCs, like proliferation, migration, α-actin expression, and Id protein levels.

The four Id proteins share a highly conserved 41-residue long HLH domain that is essential for heterodimerization with the bHLH E proteins. Synthetic peptides reproducing the four native Id HLH sequences self-associate into helix-rich structures in the low-micromolar concentration range.[11,12] In contrast, synthetic peptides reproducing the non-conserved N- and C-terminal domains (or part of them) do not adopt ordered structures.[12] However, the regions flanking the Id HLH domains seem to be important to control self-association, as only full-length Id2[13] and Id3[14,15] have been detected in the homodimeric form. Of the two helical portions in the HLH domain (Fig. 1A), only the C-terminal one has high intrinsic helix propensity, as shown by circular dichroism (CD)

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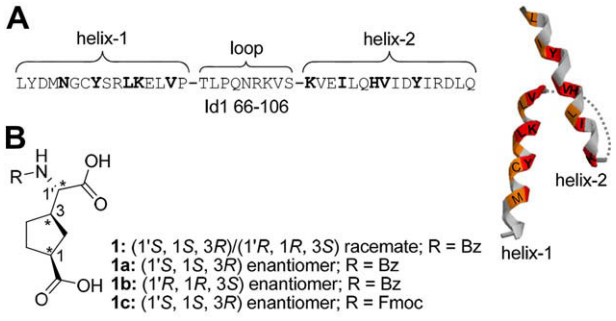

peptide construct	Cpg chirality	R	peptide chain	MW found / calc. (Da)
2	racemate	Bz	KVEILQHVIDYIRLDQ-NH_2	4217 / 4217
3	racemate	Bz	KVEILQHVIDY-NH_2	2967 / 2968
3a	(1'S, 1S, 3R)	Bz	KVEILQHVIDY-NH_2	2967 / 2968
3b	(1'R, 1R, 3S)	Bz	KVEILQHVIDY-NH_2	2967 / 2968
3a-K_6	(1'S, 1S, 3R)	Bz	KVEILQHVIDYKKK-NH_2	3738 / 3738
CF-3a-K_6	(1'S, 1S, 3R)	CF	KVEILQHVIDYKKK-NH_2	3992 / 3991
3a(scr)	(1'S, 1S, 3R)	Bz	VKEIQLVHIYD-NH_2	2967 / 2968

Figure 1. (A) Amino acid sequence of the HLH domain of Id1 (residues 66–106) and models of the helix-1 and helix-2 indicating the residues involved in intramolecular helix–helix contacts (red) and in interactions with other HLH domains (orange).[20] (B) Cyclopentylglycine (Cpg) derivatives used for the syntheses of the peptide constructs reported in (C). Bz: benzoyl. CF: 5(6)-carboxyfluorescein.

spectroscopy using two synthetic peptides reproducing helix-1 or helix-2, respectively.[16] The superior helix character of the C-terminal helix-2 might reflect its role as local structural element that triggers the folding of the HLH region, probably by assisting the otherwise quite flexible N-terminal part in adopting the correct orientation and helical conformation.[17] Besides the Id HLH one, also other HLH domains have shown a better defined C-terminal than N-terminal helix, as supported by NMR studies on the E47[18] and Max[19] HLH regions. This strengthens the importance of helix-2 as structural key element in the HLH fold.

To modulate the biological function of the Id proteins, it is important to modulate their interaction with other proteins. This may be reached with synthetic molecules that interact with and conformationally perturb the Id HLH dimerization domain. As a result, Id protein molecular recognition is inferred. The Id protein surface that recognizes and binds a bHLH protein is built from the parallel packing of helix-1 and helix-2 (Fig. 1A).[20] Considering that helix-2 is likely to play a crucial role in forming and stabilizing such helical arrangement, we made the hypothesis that a helix-2 peptide mimic might affect the folding of the HLH motif. As several examples in the literature have shown that covalent peptide dimers are better than the corresponding monomers in interacting with targeted biomolecules due to increased avidity,[21–24] we decided to use the helix-2 peptide in a covalent dimeric form deriving from the linkage of either the amino- or carboxylic ends with a bivalent linker. We recently accomplished the synthesis of N^{α}-protected *cis*-3-carboxy-cyclopentylglycines (Cpg, **1**) as conformationally constrained building blocks for the preparation of peptidomimetics (Fig. 1B).[25] Bearing two carboxylic groups, Cpg is suitable for N,N-linkage of peptide chains: therefore, we connected the Cpg unit to two copies of the hexadecapeptide reproducing Id1 helix-2 (residues 91–106), as shown in Figure 1C (peptide construct **2**). For preliminary studies on the biological properties of such peptide construct, we synthesized **2** by using racemic Cpg. The hexadecapeptide was first assembled on the solid support by Fmoc-chemistry; successively, the N-terminally free resin-bound peptide chains were crosslinked with racemic Cpg **1** that was prepared as described previously.[26] Finally, peptide construct **2** was obtained after acidic cleavage from the resin with simultaneous side-chain deprotection.

We tested the biological properties of construct **2** on the synthetic phenotype of Id-protein expressing SMCs. As these cells show α-actin downregulation, hyperproliferation and increased migration, they represent a suitable system to study potential modulators of these deregulated processes. For the cell proliferation assay,[27] cultured SMCs from human thoracic aorta were synchronized at the G_0 phase of the cell cycle and then stimulated for three days with medium containing 10% fetal calf serum (FCS) in the absence or presence of **2** (1–10 μM). We observed about 30% decrease in cell growth upon incubation with **2**. In contrast, the peptide *Ac*-(Id1 66-106)-NH_2 reproducing the full-length Id1 HLH dimerization domain stimulated cell proliferation (Fig. S3 of Supplementary data). Indeed, the Id HLH motif has been shown to inhibit the DNA binding of MyoD homodimers and MyoD-E47 heterodimers in vitro.[11]

Encouraged by the detection of a weak but still significant activity of the Cpg-crosslinked peptide construct **2**, we designed a second construct by considering the fact that, based on the homology model by Chavali et al.,[20] the N-terminal part of helix-2 (Id1 residues 91–101) is mainly involved in side-chain contacts with helix-1 (Id1 residues 70–80) (Fig. 1A). Therefore, a C-terminally truncated analog of helix-2 might be enough to mimic the helix-2 local structure. This would offer the advantage of reducing the size of the peptide construct considerably. Accordingly, we prepared peptide construct **3** by using racemic Cpg **1** and two copies of the Id1 sequence 91–101. When tested in our in vitro model, the cell response in the proliferation assay was superior in comparison to what obtained with construct **2**: indeed, as shown in Figure 2A, **3** reduced cell growth of about 40% in the low-micromolar range (~2 μM). In contrast to construct **3**, the single Id1 fragment 91–101 did not reduce cell proliferation (Fig. S4 of Supplementary data).

Peptide construct **3** modulated also cell migration, as shown by the assays performed on a modified Boyden chamber in the presence of the chemotactic agent PDGF (platelet derived growth factor): about 20% reduction of the number of migrating cells was detected (Fig. 2B). To investigate construct **3** further, we repeated then its synthesis by using the enantiomerically pure Cpg **1a** and **1b** to obtain constructs **3a** and **3b**, respectively (Fig. 1B and C). In the proliferation assay the cell responses triggered by the two constructs[28] were both comparable to the response obtained with the diastereomeric mixture **3** (Fig. 2A). This might reflect the lack of Cpg-induced preferred conformation of the peptide constructs.[29] In fact, CD measurements of **3a** (Fig. 4A) and **3b** (not shown) revealed a mostly disordered conformation, although the negative contribution to the CD spectrum between 215 and 235 nm was more significant than the one observed for the single peptide chain *Ac*-(Id1 91–101)-NH_2 (not shown), which might derive from a minor contribution of secondary structure.

Even though the peptide constructs **3a** and **3b** reduced but not blocked the cell growth, their antiproliferative effect is comparable to what obtained with other approaches reported so far to inhibit Id-protein-promoted cell proliferation, including combined antisense oligonucleotides against Id1–3[30] and cellular expression of an engineered HLH domain interacting with Id1–3[10] (the reduction of cell proliferation was about 40–60%). Moreover, the role of the Id proteins is partially overlapping during growth stimulation, as supported by the fact that the delivery of antisense molecules against

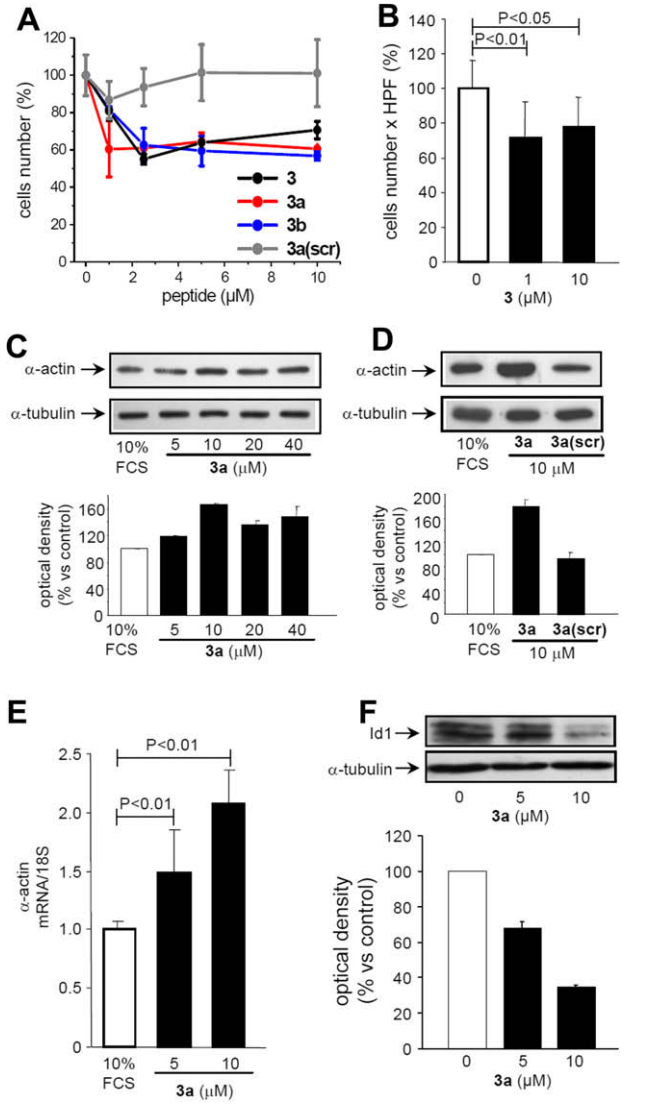

Figure 2. Modulation of proliferation (A), migration (B), α-actin protein (C, D) and mRNA (E) levels, and Id1 protein level (F) of SMCs by the peptide constructs **3** (A, B), **3a** (A, C–F), **3b** (A) and **3a(scr)** (A, D). HPF: high power field.

only one of the three Id proteins had weak[30] or no[9] effect on cell growth.

As peptide constructs **3a** and **3b** were able to negatively modulate cell proliferation and migration, we were also interested to test their effect on another deregulated process in the synthetic SMC phenotype, which is the downregulation of α-actin. As the two diastereoisomers had shown similar activity in the proliferation assay, we performed the following cellular studies with one diastereoisomer, namely **3a**. We observed a dose-dependent positive regulation of α-actin at protein as well as mRNA levels (Fig. 2C and E). As the bHLH E proteins and their inhibitors, the Id proteins, are, respectively, positive and negative regulators of α-actin,[3] the increase in α-actin mRNA level is indicative of reactivated bHLH-protein-mediated DNA transcription and of impaired Id-protein inhibitory activity on bHLH proteins. This is supported by the observation that the stimulation of α-actin by the peptide construct was accompanied by a remarkable decrease in Id1 protein levels (Fig. 2F).

Although the peptide constructs **2** and **3** do not have the characteristic features of cell-penetrating peptides, which include, for example, high content of basic amino acids (lysine and/or arginine) and residues distributed in an amphipathic pattern, they were able to enter the SMCs and influence cellular pathways. However, we thought that the introduction of a positively charged sequence motif into the peptide construct **3a** might facilitate cell penetration and intracellular nuclear addressing.[31,32] Therefore, we C-terminally elongated each peptide arm of **3a** with three lysine residues and tested the activity of the new analog (**3a-K$_6$**) on cell proliferation and α-actin protein regulation. However, despite the presence of six additional lysines, the cell response induced by **3a-K$_6$** was still comparable to the one induced by **3a** (Fig. 3A and B). Then, by using a fluorescence-labeled analog of **3a-K$_6$** containing the carboxyfluorescein (CF) moiety in place of the benzoyl group, **CF-3a-K$_6$**, we were able to monitor its dose-dependent cellular uptake and detect its predominant localization in the perinuclear region (Fig. 3C and D).

To assess the importance of the conserved Id sequence motif KVEILQHVIDY, we synthesized an analog of **3a** containing a scrambled (randomized) sequence (VKEIQLVHIYD), **3a(scr)**. The latter did not trigger cell growth inhibition (Fig. 2A) and α-actin protein expression (Fig. 2D), which indicates that the natural Id protein fragment in the peptide construct is crucial for specific molecular recognition. Accordingly, we could detect an interaction between **3a** and the synthetic peptide reproducing the Id1 HLH domain, *Ac*-(Id1 66–106)-*NH$_2$*, by CD spectroscopy. As shown in Figure 4A, the CD spectrum of the mixture containing **3a** and the Id1 HLH peptide was not superimposable to the arithmetic sum of the CD spectra of the single components, due to a positive contribution to the CD signal of the mixture in the region 190–220 nm. Moreover, for a quantitative evaluation of the binding of the peptide construct to the Id HLH domain, we synthesized a fluorescent analog of the Id1 HLH domain, which contained tryptophan in place of isoleucine-94. As such modification was structurally well tolerated,[33] we could use this analog to investigate potential interactions between the Id HLH motif and the peptide construct: indeed, both the wavelength and the intensity of the fluorescence

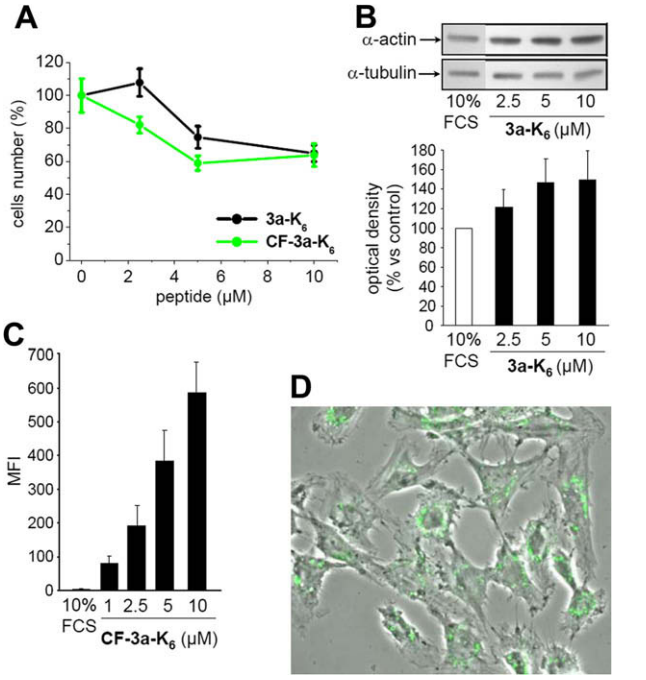

Figure 3. Modulation of SMC proliferation by constructs **3a-K$_6$** and **CF-3a-K$_6$** (A). Stimulation of SMC α-actin protein level by **3a-K$_6$** (B). Dose-dependent cellular uptake (C) and perinuclear localization (D) of **CF-3a-K$_6$**. MFI: mean fluorescence index.

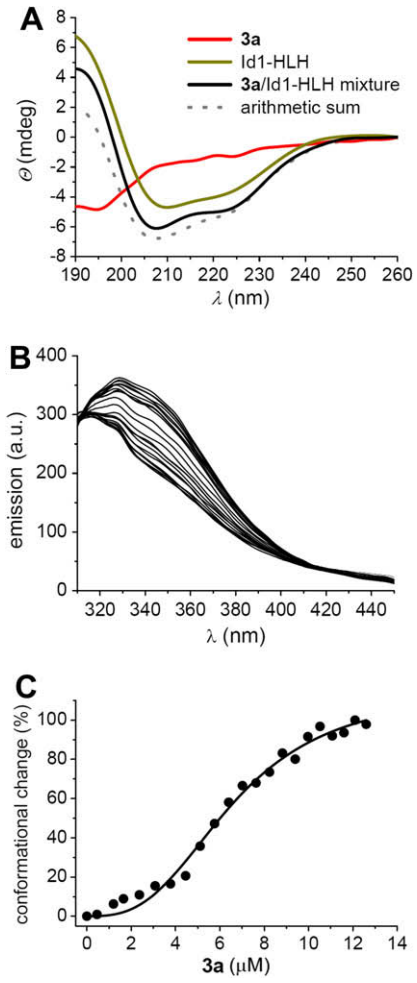

Figure 4. Interaction of peptide construct **3a** with the Id HLH domain. (A) CD spectrum of the mixture of the Id1 HLH motif *Ac*-(Id1 66–106)-NH_2 with **3a**. The CD curves of each component alone and the corresponding arithmetic sum are also shown (**3a** and Id1 HLH are in phosphate buffer 100 mM, pH 7.3, at the concentration of 90 and 30 μM, respectively). (B) Fluorescence spectra of the W/I-94 Id1 HLH analog upon addition of aliquots of a solution of **3a** (excitation at 295 nm). The emission intensity decreased by increasing concentrations of **3a**. (C) Fluorescence titration curve of the W/I-94 Id1 HLH analog with **3a**. The solid line represents the data fit obtained with the Hill equation.

emission of tryptophan are sensitive to the environment, which allows for the detection of conformational changes. A solution of the tryptophan-containing Id1 HLH analog in phosphate buffer (pH 7) was titrated with the peptide construct **3a**, and its emission was monitored upon excitation at 295 nm. Increasing amounts of **3a** led to a decrease in intensity of the maximum at 328 nm as well as of the shoulder at 345 nm. Moreover, the maximum was blue-shifted (Fig. 4B). These changes of the fluorescence spectra of the Id HLH domain confirm its interaction with **3a**, which is accompanied by a conformational change, as already indicated by the CD measurements. The titration curve based on the emission intensities at 328 or 345 nm versus the concentration of the peptide construct was fitted by using the Hill equation[34] and gave a $K_{0.5}$ value of 6.6 μM and a Hill coefficient *h* of 3 (Fig. 4C). These results show that the peptide construct recognizes the Id HLH domain and cooperatively binds the latter in the low-micromolar range.[35]

In conclusion, by using a conserved short sequence from the HLH dimerization domain of the Id proteins and a rigid scaffold for crosslinking, we developed peptide constructs that reduce SMC proliferation and migration, while stimulate α-actin mRNA and protein, a marker of a contractile phenotype of SMCs. They also decrease the Id1 protein level remarkably. Though such Id1 downregulation needs to be further investigated, it should be pointed out that, in general, Id protein expression is upregulated in proliferating cells and downregulated in differentiating cells. This correlates with our observations, as α-actin not only is a marker for a contractile SMC phenotype, but also for cell differentiation.[3] The observed inhibitory effect of the peptides on cell growth is consistent with results reported elsewhere for antisense molecules[9,30] or an engineered HLH domain expressed in the cells.[10]

Although the compounds presented here display moderate cell activity and slow cell uptake, they may contribute to the development of peptidomimetics for Id protein modulation, offering an alternative to antisense compounds.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.105.

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29. It might be reasoned that the moderate activity of constructs **2**, **3**, **3a**, and **3b** might reflect an unfavorable steric clash between the N,N-linked peptide arms. Further studies are necessary to evaluate the structural properties of the Cpg-containing peptides.

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