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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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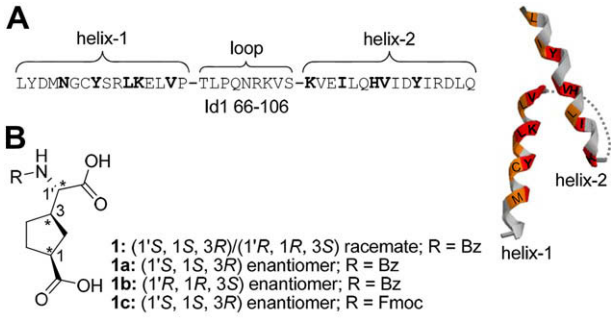

peptide construct	Cpg chirality	R	peptide chain	MW found / calc. (Da)
2	racemate	Bz	KVEILQHVIDYIRLDQ-NH_2	4217 / 4217
3	racemate	Bz	KVEILQHVIDY-NH_2	2967 / 2968
3a	(1'S, 1S, 3R)	Bz	KVEILQHVIDY-NH_2	2967 / 2968
3b	(1'R, 1R, 3S)	Bz	KVEILQHVIDY-NH_2	2967 / 2968
3a-K_6	(1'S, 1S, 3R)	Bz	KVEILQHVIDYKKK-NH_2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