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Design and synthesis of cyclic and linear peptide-agarose tools for baiting interacting protein partners of GPCRs

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Abstract—A ligation strategy for the synthesis of cyclic and linear peptides covalently linked to agarose beads designed as baits to identify new interacting partners of intracellular loops of the V2 vasopressin receptor, a member of the G-protein-coupled receptor family, is reported. The peptide-resin conjugates were subsequently shown to interact specifically with a fraction of proteins present in cellular lysates.

Cell surface receptors recognizing and responding to extracellular stimuli are the key components required for the communication between cells. G-protein-coupled receptors (GPCRs) mediate signals from a number of endogenous compounds, such as hormones, neurotransmitters, autocrine and paracrine factors, as well as having light, odorant, and taste sensing functions.[1] Therefore, GPCRs are the target for a large number of drugs and the subject of intensive research. Although remarkably diverse in sequence and function, all GPCRs share a highly conserved topological arrangement of a seven-transmembrane helical core domain joined by three intracellular loops, three extracellular loops, extracellular N-terminal, and intracellular C-terminal domains. Upon ligand binding, these receptors act as switches from inactive to active conformations able to bind and activate their intracellular partners such as the well-known G-proteins. Numerous studies have pointed out the importance of intracellular loops in G-protein coupling[1] or more generally coupling with protein partners.[2] More recently, new concepts are emerging concerning the identification of multiple membrane-associated proteins that not only govern the intracellular sequestration and/or transport of GPCR but also modulate quite dramatically GPCR ligand specificity subsequent to the cell surface delivery.[3]

In the vasopressin family, the V2 receptor subtype's i3 loop is the most fundamental determinant responsible for Gs-protein interaction and further vasopressin antidiuretic action.[4] This loop is also important for receptor localization at the endoplasmic reticulum (ER).[5] Moreover, the C-terminal tail of the V2 receptor is involved in receptor trafficking.[6] All these elements suggest the existence of receptor complexes involving other proteins than Gs and able to regulate new signalization or trafficking pathways.

Then, we decided to develop baits including the i3 intracellular loop and the C-terminal tail from the V2 receptor to identify new partners for this receptor.

Glutathione seryl transferase (GST) pull-down assays or immunoprecipitation experiments have been used to identify new GPCR binding partners by proteomic approaches.[7] An alternative would be to use peptides covalently linked to resins. Chemical modifications in the bait sequence (e.g., cyclization) may be compatible with this strategy. Chemical synthesis of peptide affinity

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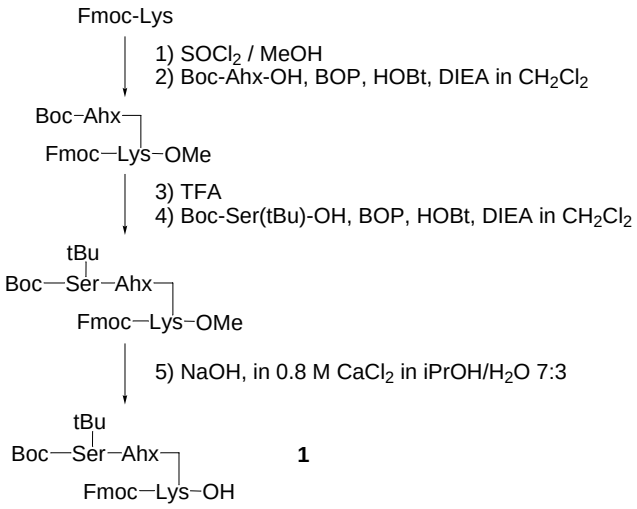
supports can provide large amounts of bait allowing an easier detection and identification of interacting proteins. Moreover, a covalent anchorage of the bait to the resin would avoid its elution with protein partners as it is the case in GST pull-down or immunoprecipitations.

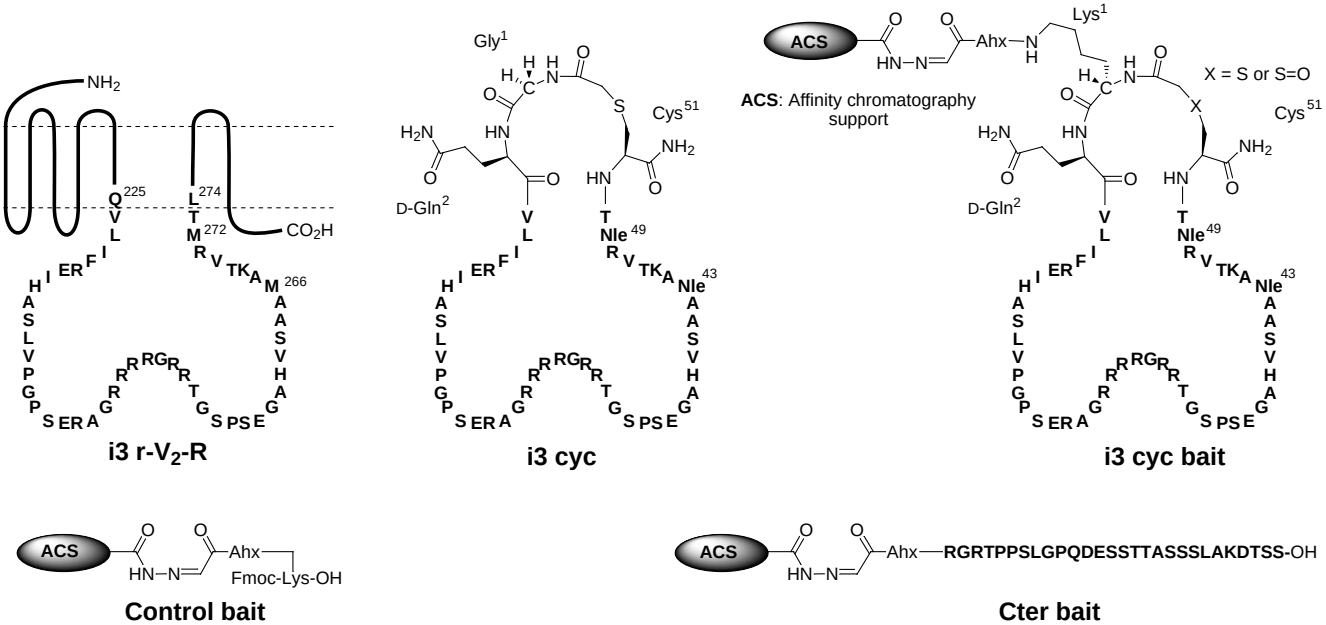
The structure of the affinity chromatography support (i3 cyc bait) used to identify proteins that could interact with the i3 intracellular loop of V_2 receptor is shown in Scheme 1. It is derived from the previously synthesized i3 cyc peptide[8] in which the Gly-1 residue of the cyclization linker is substituted for a lysine residue anchored to a solid support via its side chain. Any alteration of the i3 cyc peptide structure upon anchoring was intended to be restrained by the use of a covalent anchor selectively located on the cyclization linker and by the introduction of an aminohexanoic acid (Ahx) residue on a lysine side chain that increases the distance from the support. The affinity support bound peptide (i3 cyc bait) was prepared using solid-phase peptide synthesis followed by a double ligation strategy to cyclize a linear precursor in solution and then to anchor it to the affinity support. Supports for control experiments contained either the bare tripeptide handle (control) or a peptide derived from the C-terminal sequence of V_2 receptor (Cter).

The side-chain-derivatized lysine building block **1** was synthesized as shown in Scheme 2 (experimental information in Supplementary data). It was prepared in solution using BOP (benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate) coupling reagent from Fmoc-Lys after conversion into methyl ester. At the end of the synthesis, the methyl ester was selectively cleaved using NaOH (1.2 equiv) in 0.8 M $CaCl_2$ in iPrOH/H_2O 7:3, a reagent that is known to preserve the base-labile Fmoc group.[9]

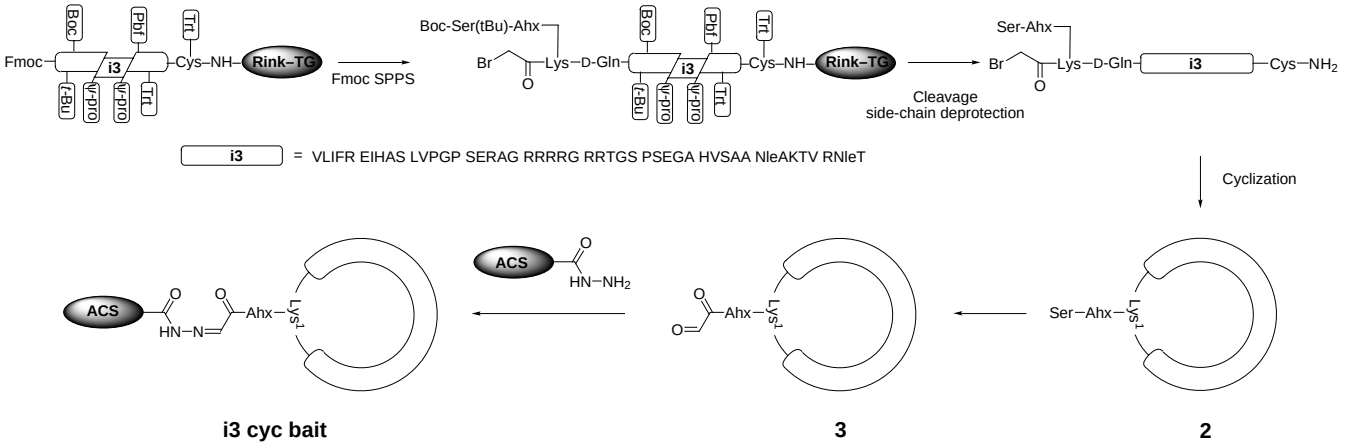


Scheme 2. Synthesis of the side-chain-derivatized lysine building block **1**.

The i3 cyc affinity resin was prepared as follows (Scheme 3). A resin bearing the Val-3–Cys-51 sequence of the peptide was synthesized as previously described for the preparation of i3 cyc peptide.[8] Fmoc-D-Gln and then the tripeptide building block **1** were coupled at the N-terminus of the protected peptide anchored on the solid phase, which was finally acylated with bromoacetic anhydride. The cyclic peptide **2** was obtained by acidolysis of the resin and cyclization by thioether ligation[10] in a phosphate buffer (Supplementary data) and then purified by HPLC. The N-terminal serine residue was then



Scheme 1. Structures of the i3 cyc peptide and of the i3 cyc bait (anchored to an affinity chromatography support, ACS) derived from the sequence of vasopressin V2 receptor third intracellular loop (i3 r-V2R). Structures of the control affinity resins Control bait and Cter bait derived from the linkage handle and of the C-terminal part of the h-V2 receptor.



Scheme 3. Structure and synthesis of the i3 cyc peptide-derivatized affinity chromatography support. Residues Ala-11–Ser-12, Val-39–Ser-40, and Lys-45–Thr-46 were introduced as Fmoc-protected pseudoproline dipeptides (ψPro).[12] Side-chain protecting groups used: tBu: *tert*-butyl (Ser, Thr, Glu), Pbf: 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Arg), Trt: trityl (His, Cys), Boc: (Lys). Supports: Tentagel® equipped with Rink amide linker (Rink-TG), affinity chromatography support (ACS).

oxidized[11] into aldehyde **3** with $NaIO_4$ in acetonitrile/water 1:1 (acetate buffer, pH 5.5). The completion of the oxidation reaction was controlled by MS (MALDI) (Supplementary data, Fig. S2), which showed the presence of the expected aldehyde **3** (60%) calculated (average) for $C_{246}H_{419}N_{87}O_{70}S$ $[M+H^+]$ 5747.6, found 5751.8. A significant part (40%) of the product displayed a mass of 5767.8 (+16), which is not unexpected since the sulfur atom of Cys residue in the cyclization linker is likely to be easily oxidized into the corresponding sulfoxide. This modification in the non-peptidic part of i3 cyc loop being unlikely to modify the structure, the solution was directly used for the derivatization of the Affi-Gel® Hz hydrazide resin (BIO-RAD) using the procedure recommended by the supplier. The progress of coupling to the resin was checked by HPLC analysis of the supernatant (Fig. 1). As the reaction proceeded (4 days), the concentration of aldehyde **3** was observed to decrease. This analytical procedure also allowed us to check that aldehyde **3** was not damaged in the medium since side products were observed neither by HPLC nor by MALDI-MS.

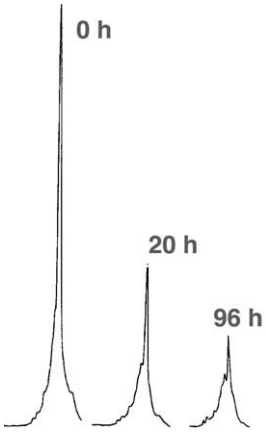
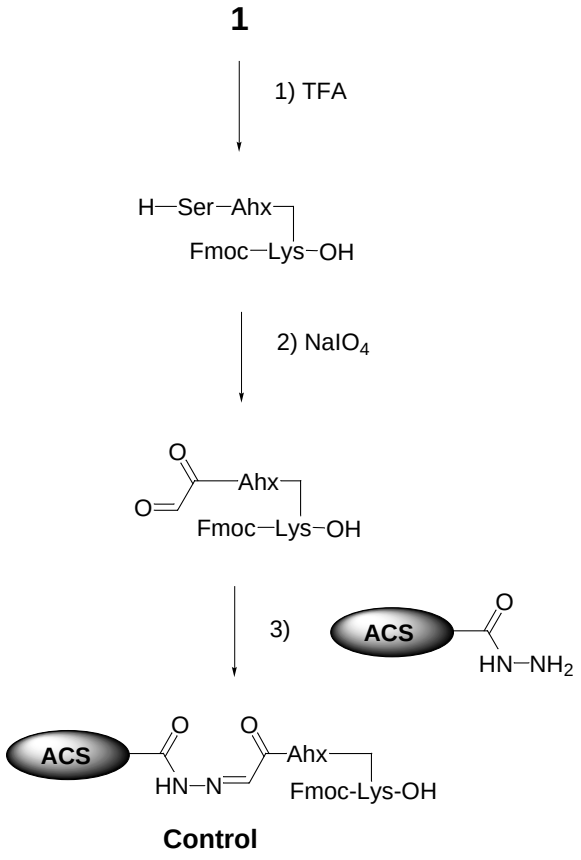


Figure 1. Grafting of the N-terminal aldehyde peptide **3** on the hydrazide resin. Evolution of the aldehyde HPLC peak in the supernatant (retention time 15.7 min, column LiChrospher 100 RP-18, buffer A 0.1% aqueous TFA, B 0.1% TFA in CH_3CN, linear gradient 20–60% buffer B over 30 min, and flow 2 mL/min).

The control affinity resin bearing the linkage agent derived from the side-chain-derivatized lysine building



Scheme 4. Structure and synthesis of the control affinity chromatography support.

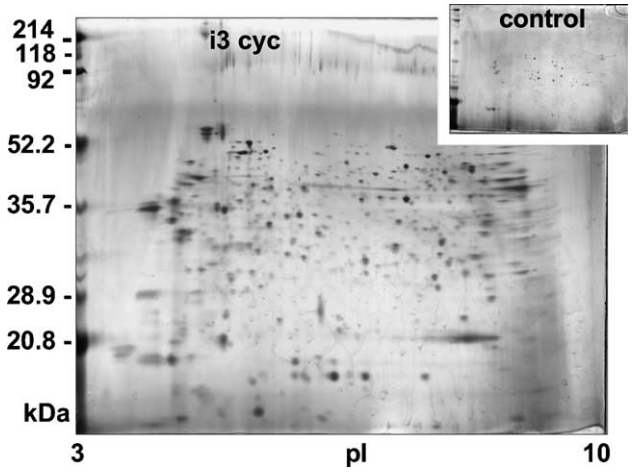


Figure 2. 2D-electrophoresis of proteins interacting with i3 cyc bait or control bait from Triton X-100 solubilized HEK lysate proteins.

block **1** only was synthesized using a similar procedure (Scheme 4).

The Cter affinity resin was prepared from the Cter peptide containing an N-terminal Ser residue, which had been previously synthesized by solid-phase peptide synthesis using Fmoc chemistry (Supplementary data). The Ser residue was then oxidized and the N-terminal aldehyde peptide Cter was grafted to the affinity gel.

The proteomic analysis to detect proteins interacting with peptide baits was performed as follows. Solubilized Triton X-100 HEK cell lysates were incubated with the different resins overnight at 4 °C. The resins were then rinsed and interacting proteins were eluted with 2D buffer to proceed to 2D electrophoresis (Fig. 2). The presence of proteins interacting specifically with the i3 cyc peptide but not with the C-terminal peptide potentially allows their identification by mass spectrometry using customary proteomic analysis procedures. The selection and identification process will be published elsewhere.

The strategy used here to anchor peptides on affinity resin bearing hydrazide groups has been shown to be compatible with three different peptides and orthogonal with the ligation procedure involving cysteine alkylation by a bromoacetyl group. It may be widely used in the synthesis of tools for the identification of protein partners using rather short peptide sequences easily accessible by chemical synthesis, but involving cyclic structures designed to mimic the peptidic segment mobility restriction present in the native protein.

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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.2005.10.061.

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