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Molecular Characterization of the NCoA-1–STAT6 Interaction

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Many protein–protein interactions involved in cell signalling, cell adhesion and regulation of transcription are mediated by short α-helical recognition motifs with the sequence Leu-Xaa-Xaa-Leu-Leu (LXXLL, where Xaa is any amino acid). Originally observed in cofactors that interact with hormone-activated nuclear receptors, LXXLL motifs are now known to occur in many transcription factors, including the STAT family, which transmit signals from activated cytokine receptors at the cell surface to target genes in the nucleus. STAT6 becomes activated in response to IL-4 and IL-13, which regulate immune and anti-inflammatory responses. Structural studies have revealed how an LXXLL motif located in 2.5 turns of an α-helical peptide derived from STAT6 provide contacts through the leucine side chains to the coactivator of transcription, NCoA-1. However, since many protein–protein interactions are mediated by LXXLL motifs, it is important to understand how specificity is achieved in this and other signalling pathways. Here, we show that energetically important contacts between STAT6 and NCoA-1 are made in residues that flank the LXXLL motif, including the underlined residues in the sequence LLPPTEQDLTKLL. We also demonstrate how the affinity for NCoA-1 of peptides derived from this region of STAT6 can be significantly improved by optimising knobs-into-holes contacts on the surface of the protein. The results provide important new insights into the origins of binding specificity, and might be of practical value in the design of novel small-molecule inhibitors of this important protein–protein interaction.

Introduction

Signal transducer and activator of transcription 6 (STAT6) is a multidomain cytoplasmic protein involved in the IL-4/IL-13 signalling cascade.[1,2] One important function of IL-4 and IL-13 is the activation of genes involved in immune and anti-inflammatory responses. Upon loss of IL-4 signalling, for example, T cells fail to differentiate into Th2 cells and B cells are unable to undergo antibody-class switching to produce IgE. Th2 cytokines and IgE are intimately involved in allergic diseases, so inhibitors of the IL-4 signalling pathway are potentially of therapeutic interest for the treatment of allergic reactions, including asthma and atopic diseases.[3]

Following binding of IL-4/IL-13 to the extracellular domain of the IL-4/IL-13 receptor, STAT6 becomes phosphorylated, whereupon it dimerizes through noncovalent interactions, translocates to the nucleus and there binds through its DNA-binding domain to the transcriptional start regions of IL-4/IL-13 responsive genes.[2] A short C-terminal segment of STAT6, called the transactivation domain, recruits components of the transcriptional machinery to activate transcription. One of these components is the essential coactivator protein NCoA-1 (also called steroid receptor coactivator-1, SRC-1).[4] The interaction between STAT6 and NCoA-1 is mediated by a short section of the STAT6 transactivation domain that includes the sequence motif LXXLL (where X is any amino acid),[5] which upon binding to a PAS-B domain of NCoA-1 folds into an amphipathic α helix. The crystal structure of a STAT6-derived peptide (794–814) complexed with NCoA-1 PAS-B domain (257–385; PDB ID code: 1OJ5, 2.2 Å resolution) was reported recently (Figure 1),[6] and revealed how the hydrophobic leucine residues that are aligned along the helix, dock onto a shallow

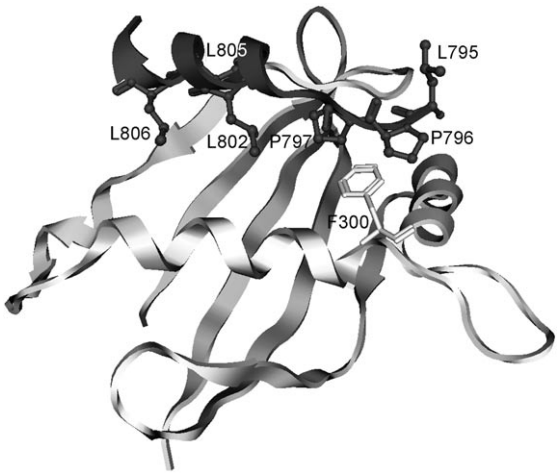

Figure 1. Ribbon diagram of the STAT6–NCoA-1 complex prepared by using PDB ID code: 1OJ5. In the helical STAT6 peptide (black ribbon; L795–E808) P797, L802 and L806 (along one face of the helix) as well as L805 (at the back of the helix) are shown in ball-and-stick format, along with L795 and P796; F300 in the NCoA-1 domain (grey ribbon) is also shown as sticks.

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hydrophobic groove on the surface of NCoA-1. This peptide was reported to bind to the NCoA domain with a dissociation constant (K_D) of 0.8 μM.

Related LXXLL motifs—also called the nuclear receptor (NR) box—participate in many other protein–protein interactions associated with different aspects of transcriptional regulation.[7–10] Crystal structures of several protein–protein interactions that involve LXXLL motifs have revealed broadly similar mechanisms of recognition of the amphipathic helical motif.[9] This raises important questions about the origins of specificity observed in these interactions. For example, NCoA proteins each contain several LXXLL motifs (NR boxes) as well as domains that can bind to them. Nevertheless, only NCoA-1 interacts with STAT6, although many of the residues that line the LXXLL-binding site of the PAS-B domain are conserved throughout the NCoA family.[6] Evidence exists for some NR boxes that residues that flank the LXXLL motif make important contributions to the specificity code.[8,11]

In the case of STAT6, mutagenesis of L802 and L805 to alanine (which corresponds to LXXLL, L802/805 underlined) abolished the interaction with NCoA-1 in both in vitro GST pull-down assays and in vivo.[5] In this work, we report more detailed studies of the STAT6–NCoA-1 interaction, which have revealed additional residues that flank the LXXLL motif in STAT6 that are important for binding to NCoA-1. We also report a new fluorescence-polarization assay, which should be of value for discovering inhibitors of this interaction. As a first step towards this goal, we also demonstrate how the affinity of peptides derived from this region of STAT6 can be significantly improved by optimising knobs-into-holes contacts on the surface of the NCoA-1 protein.

Table 1. STAT6-derived peptides and their affinities for NCoA-1. The dissociation constants (K_D) or inhibition constants (K_i) were determined by direct FP or competitive FP assay, respectively, as described in the Experimental Section.

Peptide	Sequences	Molecular mass calcd [M+H]	Molecular mass ES-MS [m/z][c]	HPLC[d] t_R [min]	K_D (K_i) [μM]
STAT6	GTWIGEDIFPPLLPPTEQDLTKLLLEGQGESG[a]				
1	LLPPTEQDLTKLLLEGQGESGY	2401.2	2401.1	15.6	0.26
2	Fluo-LLPPTEQDLTKLLLEGQGESG[b]	2596.2	2596.3	16.0	0.32
3	LPPTEQDLTKLLLEGQGESGY	2288.1	2288.1	12.2	7.5
4	Fluo-GTWIGEDIFPPLLPPTEQDLTKLLLEGQGESG[b]	3809.1	3808.8	21.0	0.04
5	Ac-GTWIGEDIFPPLLPPTEQDLTKLLLEGQGESG[b]	3492.8	3492.8	22.9	0.04
6	Ac-GTWIGEDIFPPALPPTEQDLTKLLLEGQGESG[b]	3450.7	3450.5	21.9	0.62
7	LLPPTEQDLTKLLLY	1757.0	1757.0	18.6	0.20
8	LLPPTEQDLTKLLY	1643.9	1643.8	17.1	0.80
9	Fluo-GTWIGEDIFPPLLPPTEQD[b]	2504.3	2504.1	16.8	>10

[a] Sequence of STAT6 protein from residue 783 to 814. [b] Fluo- and Ac- correspond to *N*-(5,6)-carboxyfluorescein and *N*-acetyl groups, respectively. [c] The mass corresponds to the monoisotopic $[M+H]^+$ species observed by MALDI-MS. [d] HPLC analyses were performed by using a Vydac 218TP54 C18 column (250×46 mm; particle size 5 μm; solvent A: H_2O/TFA (0.1%, *v/v*); solvent B: MeCN/TFA (0.1%, *v/v*); flow rate 1 mL min^{-1}; linear gradient A/B: from 85:15 to 45:55 in 25 min); t_R: retention time.

Results

Fluorescence polarization (FP) binding assay

A linear peptide, including residues L794–G814 of STAT6, was first chosen for study since this includes the key LXXLL motif and all of the residues visible in the STAT6–NCoA-1 crystal structure (PDB ID code: 1OJ5, namely L795–E808). Peptide **1** (Table 1) has an additional C-terminal tyrosine to aid concentration measurements by UV. Peptide **2** was synthesised with solid phase methods by using Fmoc chemistry, and (5,6)-carboxyfluorescein was coupled at the N terminus. The human NCoA-1 fragment used for these studies included residues T257–R385, which was expressed as an N-terminal His_6-tagged protein in *E. coli*. After removal of the His-tag by proteolysis, three additional residues (GHM) remain at the N terminus of the T257–R385 NCoA domain. This protein, which was homogeneous by SDS-PAGE, gave the expected mass by ES-MS, and was used for all the studies described below.

Titration of peptide **2** (1 μM final concentration) with the NCoA-1 protein (0 to 14 μM) was performed in HEPES buffer in black 96-well microtiter plates. Figure 2A illustrates a nonlinear fit of the FP data to a single-site binding model, which gives a $K_D = (320 \pm 20)$ nM for this interaction, with a fluorescence polarization dynamic range $\Delta mP = (103 \pm 6)$ *mP* (*mP* of bound peptide − *mP* of free peptide).

Next, competitive FP assays were performed by using peptide **2** (1 μM), the NCoA-1 domain (5 μM) and serial dilutions of peptide **1** as competing ligand in 96-well microtiter plate format in HEPES buffer. The IC_{50} value of 8.3 μM was determined from the dependence of the FP signal on peptide **1** concentration (Figure 2B). Thereafter, the inhibition constant (K_i) value of the competitive inhibitor (**1**) was calculated to be (260 ± 20) nM by using the method described by Nikolovska-Coleska et al.[12] This is close to the K_D value obtained for peptide **2** as determined by direct FP, and suggests that the fluorophore in **2** does not have a major influence on the interaction with NCoA-1. Finally, the dissociation constant of peptide **1** binding to the NCoA-1 domain was also determined by isothermal titration calorimetry (ITC). Three independent ITC measurements at 298 K gave $K_D = (443 \pm 62)$ nM, $\Delta H = (-7.63 \pm 0.13)$ kcal mol^{-1} and $T\Delta S = +1.03$ kcal mol^{-1}, after data fitting to a simple 1:1 interaction model (Figure 2C).

Structure–activity studies

Each residue in peptide **1** was replaced in turn by alanine and the affinity of the resulting peptide for NCoA-1 was determined by FP. The results revealed that three changes, at P797, L802 and L806 (Table 2 and Figure 3) essentially abolished the interaction with NCoA-1 ($K_i > 25$ μM), whereas mutation of

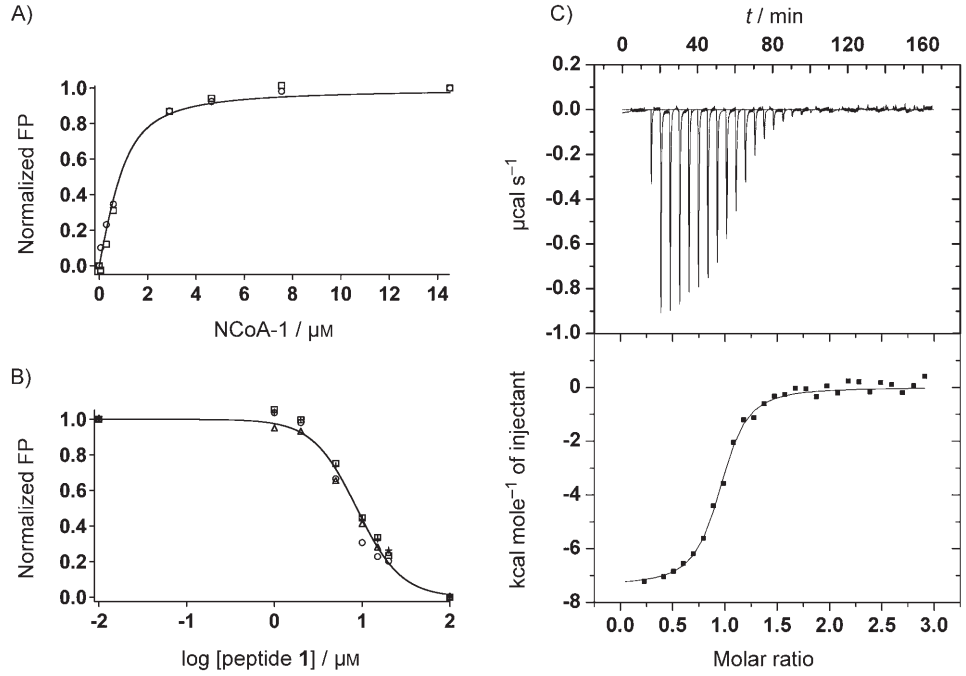

Figure 2. A) Binding of peptide **2** (1 μM) to NCoA-1 (0–14 μM) monitored by FP; $K_D = (320 \pm 20)$ nM. B) Competition FP assay with peptide **1** as competing ligand (0–100 μM). The concentrations of peptide **2** and NCoA-1 were fixed at 1 μM and 5 μM, respectively. The experiment was repeated four times, and gave an IC_{50} of 8.3 μM and a K_i of (260 ± 20) nM. C) ITC for peptide **1** (500 μM) binding to NCoA-1 (30 μM) at 25 °C. Top panel: raw data from the titrations; with the exception of the first peak, all peaks correspond to 8 μL injections. Bottom panel: integrated curves were fitted with the ORIGIN software by using a single-site binding model.

L794 led to a ≈50-fold reduction in affinity, and the effects of changes at T798 and L805 were smaller but still significant (ca. 13–14-fold weaker). All other mutations had only minor effects on affinity.

To confirm the importance of L794, additional experiments were performed. First, peptide **3**, which lacked L794 (Table 1), was prepared and assayed, and was found to bind NCoA-1 with about 30-fold reduced affinity. Secondly, a longer STAT6-derived peptide that encompassed residues 783–814 was studied. Peptide **4**, which had a fluorescein tag, and peptide **5** (without the tag) bound the NCoA-1 domain with about six- to sevenfold improved affinity ($K_D \approx 40$ nM) compared to **1** and **2** in direct and competitive FP-binding assays. However, the L794A mutant (peptide **6**) again bound NCoA-1 with a significantly reduced affinity ($K_D \approx 0.62$ μM). Finally, although the C-terminally truncated peptides **7** and **8** retained significant affinity for NCoA-1, peptide **9** showed much reduced affinity.

The plasticity of the van der Waals contacts with NCoA-1 at two key sites in the STAT6 binding pocket was also explored with analogues of peptide **1**, in which either L802 or L806 were exchanged for a variety of similar hydrophobic residues. The results show that substitution of leucine by valine at both sites led to a significant drop in affinity, whereas isoleucine, norleucine (Nle) and phenylalanine were all well tolerated (Table 2). However, substitution of cyclohexylalanine (Cha) at position 806 led to a tenfold increase in affinity; this was presumably due to an optimisation of the van der Waals contacts.

Table 2. Mutagenesis studies with peptide **1**. The mutations and the resulting affinity (K_i) to the NCoA-1 domain are shown, as measured by competitive FP.

Peptide[a]	K_i [μM]	Peptide[a]	K_i [μM]	Peptide[a]	K_i [μM]
peptide **1**	0.26 ± 0.02	K804A	0.39 ± 0.04	L802V	1.08 ± 0.1
L794A	**13.3 ± 0.6**	L805A	3.44 ± 0.2	L802I	0.32 ± 0.02
L795A	0.52 ± 0.03	**L806A**	**> 25**	L802Nle	0.22 ± 0.02
P796A	0.39 ± 0.04	L807A	0.27 ± 0.04	L802F	0.30 ± 0.04
P797A	**> 25**	E808A	0.19 ± 0.1	L802Cha	0.94 ± 0.06
T798A	3.67 ± 0.35	G809A	0.14 ± 0.05	L806V	3.1
E799A	0.78 ± 0.01	Q810A	0.16 ± 0.01	L806I	0.32 ± 0.02
Q800A	0.11 ± 0.02	G811A	0.21 ± 0.02	L806Nle	0.68 ± 0.04
D801A	0.35 ± 0.1	E812A	0.18 ± 0.03	L806F	0.47 ± 0.04
L802A	**> 25**	S813A	0.13 ± 0.02	**L806Cha**	**0.03 ± 0.015**
T803A	0.38 ± 0.04	G814A	0.28 ± 0.03		

[a] The normal amino acid code is used; Nle = L-norleucine and Cha = L-cyclohexylalanine. All peptides were > 95% pure as determined by analytical HLPC and 600 MHz ^{1}H NMR spectroscopy, and gave the masses expected by MALDI-MS (for details see Table 1 legend).

Discussion

Fluorescence polarization (FP) is a sensitive, robust and frequently used method for the study of protein–ligand interactions.[13,14] The successful use of FP re-

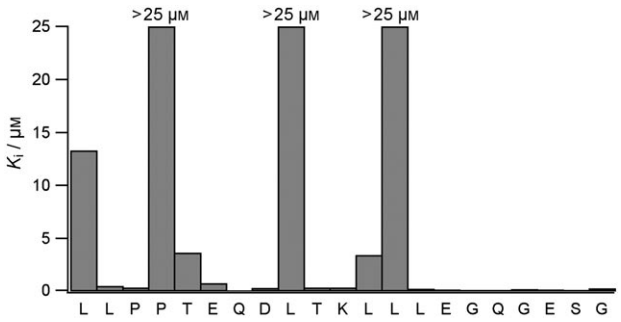

Figure 3. Summary of the binding affinities of a panel of peptides for NCoA-1 that resulted from substitution of each residue in the sequence 794–814 (LLPPTEQDLTKLLLEGQGESG; the LXXLL motif is underlined) in turn by alanine (Table 2). The K_i values from competition FP assays are indicated.

quires a fluorescently labelled ligand with high affinity for the receptor of interest. The fluorophore should not influence ligand binding, but should have an appropriate fluorescence lifetime and cause minimal unspecific binding. In this case 5(6)-carboxyfluorescein was appended to a linear peptide that included residues L794–G814 of STAT6, since this region includes the key LXXLL motif and all of the residues visible in the STAT6–NCoA-1 crystal structure (PDB ID code: 1OJ5), namely L795–E808. The apparent dissociation (K_D) of peptide **2** for NCoA-1 was (320 ± 20) nM by FP, whereas the corresponding unlabelled peptide **1** showed a K_D of 443 nM by ITC measurements. The good agreement with the results reported earlier[6] strengthen confidence in these affinity determinations by FP and ITC.

In the earlier studies of Litterst and Pfitzner,[5] only L802 and L805 were mutated in turn to alanine, and these mutations abolished binding of STAT6 to NCoA-1 in their GST pull-down assays. To extend these studies, we systematically replaced each residue in peptide **1** by alanine, and determined the effects on binding to the NCoA-1 domain using the competitive FP assay. In addition, binding assays were performed with both longer and shorter peptides (Table 1), which showed that residues downstream of L806 do not contribute significantly to binding. However, residues on the N-terminal side of the LXXLL motif in STAT6 clearly make important energetic contributions to binding NCoA-1. In particular, the side chains of L794, P797, L802 and L806 are key hot-spot residues in this interaction, in which T798 and L805 are of intermediate importance.

The crystal structure of the STAT6 peptide–NCoA-1 domain complex (PDB ID code: 1OJ5) provides a rationale for some of these results, since P797 and the side chains of L802 and L806 are deeply buried at the interface, whereas the side chain of L805 points to the side of the interface and is partly solvent exposed (Figure 1). The P797A substitution will also alter the conformational and hydrogen-bonding properties of the peptide, as well as the van der Waals contacts with the base of the binding site, and the importance of these individual changes on binding affinity are presently difficult to quantify. However, L794 is not visible in the STAT6–NCoA-1 crystal structure. The N-terminal residue of STAT6 included in the structure is L795. The ϕ/ψ angles of residues L795, P796, P797 and T798 are in the extended (β) region, which clearly suggests that L794 is not part of the helical epitope that encompasses the LXXLL motif. Indeed, it seems the backbone around L795 must kink (or turn) to allow the side chain of L794 to fold back against a hydrophobic patch on the surface of NCoA-1 near F300, rather than extending out into solution.

Finally, the plasticity of the STAT6 binding site on NCoA-1 was explored at two key sites with a series of peptide **1** analogues in which either L802 or L806 were exchanged for a variety of similar hydrophobic residues. The results show that substitution of leucine by other aliphatic and even aromatic groups is tolerated without a significant drop in affinity (Table 2). Most interesting is the effect of substituting leucine by cyclohexylalanine at position 806, which led to an about tenfold increase in affinity; or expressed another way: addition of a whole cyclohexane ring, in going from alanine to cyclohexylalanine, improved the affinity by over 1000-fold (Table 2). Molecular modelling studies suggest that the larger cyclohexylalanine side chain fills a cavity that is left partially empty by the L806 side chain in peptide **1** (Figure 4). This increased hydrophobic contact surface appears to be a major source of the improved binding affinity.

The binding studies described here considerably extend our understanding of specificity in this important STAT6–NCoA-1 interaction, and establish which side chains displayed along the backbone of the STAT6 peptide are important for binding to NCoA-1. This information could also be of practical value, as there is growing interest in the design of novel protein–protein interaction inhibitors in general, and of the STAT6 signalling pathway in particular. Thus selective inhibitors of some steroid receptor–coactivator interactions have been designed by using helix-stabilized cyclic peptides,[15–18] as well as alternative nonpeptidic scaffolds.[19,20] A careful analysis of energetically important interactions and potential knobs-into-holes inter-

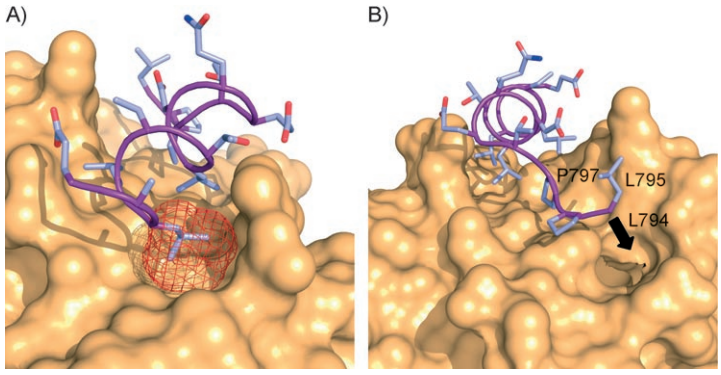

Figure 4. The STAT6–NCoA-1 structure from RCSB PDB file 1OJ5. The peptide is shown as a ribbon and stick model, and the surface of NCoA-1 is shown in beige. A) View from the C terminus of STAT6, with the L806 side chain and the pocket filled by the cyclohexylalanine mutant (L806Cha; Table 2); the surface of a cyclohexyl side chain is indicated by the red mesh. B) View from the N terminus of STAT6, showing P797 and the likely binding site for L794.

actions, inside and outside such LXXLL binding sites, might provide new opportunities for the design of novel specific transcription factor inhibitors.

Experimental Section

Description of the synthesis and analytical characterisation of the peptides, as well as the production of the NCoA-1 protein used in this work are given in the Supporting Information.

Fluorescence polarization (FP) assay: In all experiments HEPES buffer was used (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, pH 7.4). Assays were carried out in black 96-well microtiter plates (Greiner). For the K_D measurement of peptide **2**–NCoA-1 complex, solutions that contained peptide **2** (10 μL stock solution, final concentration 1 μM) and NCoA-1 (final concentration 0–14 μM) were prepared in a total volume of 100 μL HEPES buffer. The plate was shaken for 5 min at room temperature, and FP was then measured by using the standard configuration. After normalisation, the fraction of bound peptide **2** (B) was determined, and the K_D was calculated according to Equation (1):

$$B = \frac{1}{2[R_t]}\left[(L_t + R_t + K_D) - \sqrt{(L_t + R_t + K_D)^2 - (4 L_t R_t)}\right] \quad (1)$$

where L_t is the total peptide (ligand) concentration, R_t is the total protein (receptor) concentration and K_D is the dissociation constant.[14]

Competition FP: Each well of a black 96-well microtitre plate was loaded with 10 μL of 2 μM (or 10 μM for alanine scan experiment) peptide **2** solution, 10 μL of 10 μM (50 μM in the alanine scan) NCoA-1 solution, and with peptide **1** (or analogue) at a final concentration in the range 0–100 μM (or 0–400 μM for weak binders). In each well the total volume was made up to 100 μL by the addition of HEPES buffer. The microtiter plate was incubated at room temperature for 5 min, and the FP was then measured; each measurement was duplicated. In all assays, peptide **1** was included in the first two rows as a positive control. Since the total fluorescence intensity of peptide **2** remains similar for all samples, the fraction of peptide bound to NCoA-1 is correlated to the fluorescence polarization (F_p). Thus, the free fraction (L_f) of peptide **2** can be derived from Equation (2):

$$L_f = \frac{F_p - F_p^f}{F_p^b - F_p^f} \quad (2)$$

in which F_p^f corresponds to the FP of the total free peptide (0% inhibition) and F_p^b corresponds to the FP of the total bound peptide (100% inhibition).

After normalisation, data were fitted with IGOR pro software (WaveMetrics, Lake Oswego, OR, USA) to a sigmoid equation to determine the IC_{50} values. The K_i values were calculated from IC_{50} values according to the method described by Nikolovska-Coleska et al.[12]

Isothermal titration calorimetry (ITC): Experiments were performed by using a Microcal VP-ITC instrument. Both injected peptide **1** and NCoA-1 were equilibrated in ITC buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA). NCoA-1 was dialyzed into ITC buffer and used at a final concentration of 30 μM. Experiments were carried out at 298 K and typically involved 30 injections of peptide **1** (8 μL; 500 μM). The results were analyzed with the manufacturer's software by using a simple 1:1 ligand-binding site model.

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Keywords: fluorescence polarization · helical epitopes · peptides · protein–protein interactions · receptors

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