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Mapping of protein-protein interactions between c-myb and its coactivator CBP by a new phage display technique

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Abstract We have developed a phage display technique for the mapping of protein-protein interaction sites and characterized the interaction between the c-myb proto-oncogene product and its co-activator CBP. Arbitrary DNA segments of the c-myb gene were cloned into a modified phagemid which allowed for expression in all possible reading frames. The mini-library encompassing all functional domains of the protein was propagated as phages and screened with different bait proteins. Alignment of the sequences revealed that the amino acids 317–342 of Myb interact with the CBP protein. Furthermore, an intramolecular interaction of the N-terminal Myb DNA binding domain with the C-terminus (amino acids 541–567) could be detected.

Key words: Phage display; Protein-protein interaction; c-Myb; CBP

1. Introduction

In order to understand complex reactions in molecular biology such as signal transduction pathways or the transcriptional machinery, it is necessary to characterize protein-protein interactions involved in these processes. Two-hybrid assays and transient transfection assays are established methods to study these interactions. While in vivo systems are prone to false positive signals due to dominant non-specific interactions of the biological background, we investigated whether a phage display system could be an alternative to the eukaryotic systems. In contrast to the methods listed above, the phage display system is based on in vitro selection and amplification cycles, which lead to the enrichment of interaction partners. Filamentous phage display [1] of peptides, protein fragments and proteins is a powerful method to select functional clones from vast libraries.

This technique exploits the unique features of the phage life cycle (review: [2]): genes can be cloned into the phagemid polylinker which will then be expressed as heterologous fusion proteins with the g3p protein. The recombinant phages will display one or more copies of these fusion proteins on their tips. Therefore, peptides [3], antibody fragments (review: [4]), or protein domains [5] may serve as a starting population for successive selection cycles, in which affinity binding to specific ligands is used to select and enrich for optimal binding partners in the pool. Since the phenotype expressed on the phage surface directly corresponds to the genotype of the phage, the active binding fraction contains the corresponding sequence information. The DNA sequence of individual phages can then be determined and an amino acid consensus sequence of the binding domain can be translated from the alignment of several sequences. We have improved this technique by a cloning strategy which allows shotgun cloning of arbitrary sequences into all possible reading frames of the phagemid vector.

We were interested in the characterization of the interaction between Myb proteins (review: [6]) and their cofactors and used a phage display technique to map domains which are involved in this process. The proto-oncogene c-myb encodes a DNA binding protein of approximately 75 kDa. The three members of the mammalian Myb family A-myb, B-myb and c-myb recognize the DNA sequence AACNG with high specificity and seem to be modulated in their binding capacity by cell-cycle specific phosphorylation. A DNA binding domain, a transactivation domain, a negative regulatory domain and several phosphorylation sites have been determined in structural studies of the protein. It was reported recently that the transacting activities of the protein are regulated by the C/EBPβ (NF-M) protein [7,8] and the bridging factor CBP [9,10].

We have applied the phage display system in order to analyze the interaction of the Myb protein with its cofactor CBP. Furthermore, we have studied with this technique the intramolecular contact between the N- and C-termini of the Myb protein.

2. Materials and methods

2.1. Bacterial strains

TG1: K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*Δ5/F′, *tra*Δ36, *pro*A$^+$B$^+$, *lac*I^q, *lac*ZΔM15

HB2151: K12, Δ(*lac-pro*), *ara*, NaI^r, *thi*/F′, *pro*A$^+$B$^+$, *lac*I^q, *lac*ZΔM15

LK111(λ): $r_k^-m_k^-$, *thi*-1, *thr*-1, *leu*B6, *ton*A21, *sup*E44, *lac*I^{q}ZΔM15, Hfr, λ$^+$

DH5a: *end*A1, *hsd*R17(rm_k^-, m_k^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A(NaI^r), *rel*A1, Δ(*lac*ZYA-argF)$_{U169}$, (m80*lac*ZΔM15)

2.2. Vectors and helper phages

Phagemid pCANTAB5E and the expression vector pGEX-2T were purchased from Pharmacia, the plasmid pQE30 was bought from QIAGEN. The helper phages M13K07 and VCSM13 were obtained from Pharmacia and Stratagene respectively.

2.3. Modification of the pCANTAB5E phagemid

All synthetic oligodeoxynucleotides and cloning linkers were synthesized on a Milligen Cyclone DNA synthesizer, the reagents were purchased from MWG-Biotech, Ebersberg. Using standard protocols [11], the oligodeoxynucleotides shown in Fig. 1 were hybridized and

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ligated into the pCANTAB5E phagemid which had been previously digested with the *Sfi*I and *Not*I restriction endonucleases. The new vectors were verified by dideoxy sequencing.

2.4. Fragmentation of linear DNA

In the presence of Mn^{2+} ions, DNase I produces mostly blunt-ended fragments [12]. DNase I (Sigma) was stored aliquoted (c = 1 mg/ml) in 0.01 N HCl. 1 μg of linear DNA was digested with various amounts of DNase I at 15°C for 10 min in a buffer consisting of 50 mM Tris-HCl, pH 7.5, 2 mM $MnCl_2$, 100 μg/ml bovine serum albumin. The reaction was stopped by adding EDTA to a final concentration of 10 mM. Under non-optimal conditions (excess enzyme, high glycerol content), mung bean nuclease cleaves double-stranded DNA unspecifically. 1 μg of linear DNA was digested with 10 U of mung bean nuclease (NEB) at 37°C for 10 min in a buffer consisting of 30 mM sodium acetate, pH 5.0, 50 mM zinc acetate, 0.2 mM cysteine, 0.1% Triton X-100 (w/v), 50% glycerol (v/v). The reaction was stopped by adding EDTA to a final concentration of 10 mM. All fragments generated by mung bean nuclease or DNase I digestion were treated with T4 DNA polymerase. The polymerase and the 5′-3′ exonuclease activity of the enzyme removes cohesive ends or single strand breaks. The restriction endonucleases *Hae*III, *Rsa*I, *Alu*I, *Bst*UI and *Bsu*RI recognize four base sequences and produce blunt-ended fragments. The enzymes were purchased from NEB or MBI-Fermentas and were used following the suppliers' guidelines. The reaction was stopped by adding EDTA to a final concentration of 10 mM.

2.5. Immobilization of the proteins

Recombinant proteins were expressed in bacteria and purified using standard protocols. The proteins were immobilized on 96-well microtiter plates (Nunc-ImmunoSorb) or on Ni^{2+}-NTA-*HisSorb* strips (Qiagen). 200 μl of the purified protein (10 μg/ml in phosphate buffered saline (PBS) and 15% glycerol (v/v)) was incubated overnight at 4°C in the microtiter wells. The supernatant was removed and the wells were blocked with blocking buffer (1×PBS, 1% bovine serum albumin (w/v) and 0.1% Triton X-100 (v/v)). Approximately 250 ng of His-tagged protein per well was immobilized on Ni^{2+}-NTA-*HisSorb* strips overnight at 4°C in 1 PBS. Unspecific binding was blocked with 300 μl blocking buffer.

2.6. Panning of recombinant phages

1×10^9 pfu/ml of recombinant phages in 200 μl blocking buffer were incubated per well at 4°C for 4 h with the immobilized proteins. Depending on the stringency, up to 30 washing steps were performed with blocking buffer. For the reinfection of *Escherichia coli* TG-1 cells, 200 μl of a TG-1 cell suspension (OD_{600} = 0.5) was added and incubated at 37°C for 30 min.

2.7. Phage rescue of recombinant phages

Transformed cells (800 μl) were incubated at 37°C in 15 ml 2×YT-AG until a cell density of OD_{600} = 0.5 was reached. The suspension was then infected with 3×10^{10} pfu of M13K07 helper phages and shaken for 1 h at 37°C. The cells were pelleted at 1000×*g* for 10 min, suspended in 2×YT-AK and incubated overnight at 37°C. The culture was centrifuged at 1000×*g* for 20 min and the supernatant was precipitated with 1/5 volume of PEG/NaCl (20% polyethylene glycol 6000 (w/v), 2.5 M NaCl) for 4 h at 4°C. The phages were harvested at 6000×*g* for 4 h. The visible pellet was dissolved in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl and 10 mM $MgSO_4$. The suspension was sterile filtered (0.45 μm filter) and stored at 4°C.

2.8. Infection of TG1 cells with recombinant phages

TG1 cells were grown in 20 ml 2×YT medium at 37°C to a cell density of OD_{600} = 0.5. A suspension of recombinant phages was added and the culture was incubated at 37°C for 1 h. Phage rescue was performed as described above.

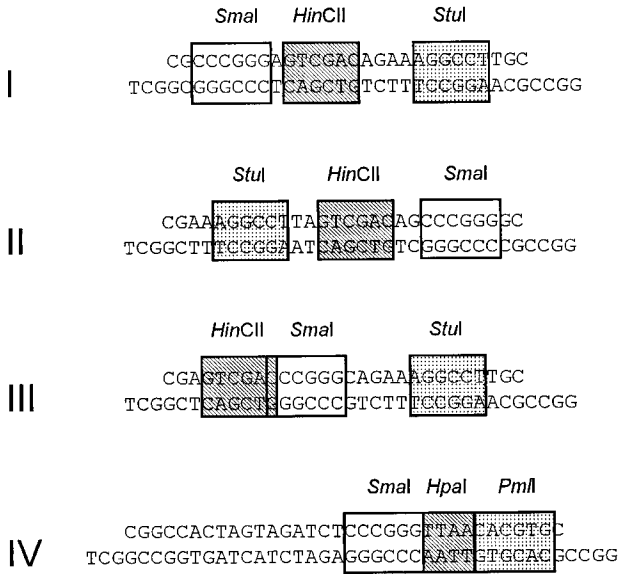

Fig. 1. Design of the oligodeoxynucleotides used for the modification of pCANTAB5E. Open boxes indicate reading frame +0, shadowed boxes reading frame +1, stippled boxes reading frame +2.

3. Results

3.1. Construction of the phagemid

We first modified the pCANTAB5E phagemid, by introducing multiple blunt restriction sites between the *pel*B leader sequence and the g3p gene. The double-stranded oligodeoxynucleotides I–IV (Section 2) were ligated into the *Sfi*I and *Not*I and the resulting plasmids were verified by sequencing. The reading frame between the leader sequence and the g3p protein must stay intact, as otherwise stop codons or frameshifts will lead to non-functional g3p coat proteins. The design of these polylinkers (depicted in Fig. 1) allows generation of different reading frames and raises the probability of inserting arbitrary gene fragments into the appropriate reading frame. Furthermore, the choice of blunt-ended restriction sites permits direct cloning of gene sequences.

3.2. Construction of a c-myb library

With these modified phagemids we generated a mini-library of the c-myb gene. Considering the fact that most of the known protein domains rarely involve more than 100 amino acids, we decided to utilize DNA fragments of an average length of 300 bp for the library. Furthermore, DNA sequences shorter than 1000 bp should circumvent incorrect folding of

Table 1
Compilation of clone numbers from three successive selection experiments

Protein	Number of clones recovered		
	First selection cycle	Second selection cycle	Third selection cycle
CBP1	2.4×10^3	3.4×10^3	9.4×10^3
CBP2	1.6×10^4	8.0×10^4	1.5×10^5
CBP3	6.4×10^3	2.4×10^3	6.0×10^3
BSA	3.8×10^3	8.0×10^3	2.4×10^3

CBP1, CBP2, CBP3 were bait proteins and BSA was used as a control.

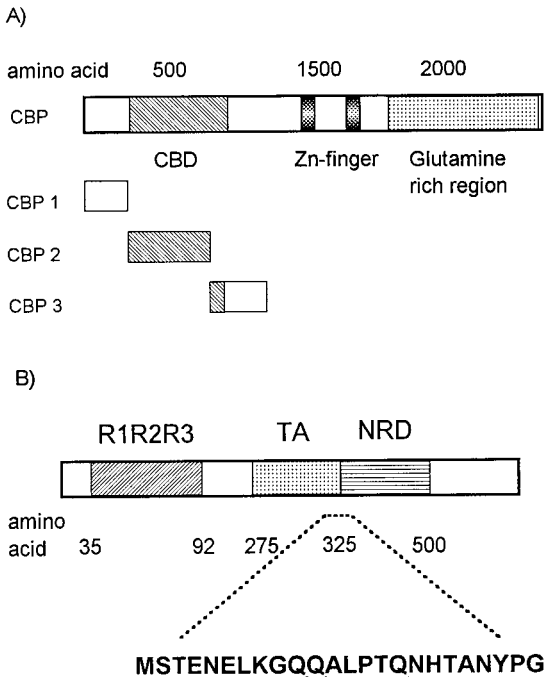

Fig. 2. A: Schematic drawing of the CBP protein and the truncated proteins used as bait. CBP harbors an N-terminal CREB binding domain (CBD), a zinc finger motif and a C-terminal glutamine rich region. The CBP2 protein encompasses the main portion of the CREB binding domain, whereas the other bait proteins CBP1 and CBP3 do not. B: Location of the amino acid sequence in MYB selected by the CBP2 protein. This sequence is depicted by an insert of amino acids in single letter code and situated between the transactivating (TA) and negative regulatory domain (NRD). The three repeats of the N-terminal Myb DNA binding domain are represented by R1, R2 and R3 respectively.

the resulting fusion protein [13]. The c-myb gene was fragmented either by limited DNase I treatment, mung bean nuclease, or restriction endonuclease digestion, and the length distribution was monitored on agarose gels. We made use of all three methods for the construction of the library. Furthermore, the restriction enzymes were permutated in successive digestion reactions for the purpose of generating of possible 5′- and 3′-ends. Each insert preparation was ligated separately into the three reading frames of the four vectors. Due to the low yield of blunt end ligation, 100 ng dephosphorylated linear phagemid and 100 ng random cut c-myb DNA resulted in 10^3–10^4 clones per reaction. In order to make certain that the library was complete, up to 100 separate ligations were mixed to generate a pool of 10^5 independent clones.

3.3. Interaction between Myb and CBP

It has been shown recently by in vitro and in vivo studies that the co-activator CBP can interact with the Myb protein and stimulates transcription [9],[10]. We therefore asked whether our phage c-myb library could be used to map the precise location of the Myb domain interacting with CBP. We utilized three truncated CBP proteins of an average length of 400 amino acids (CBP1, amino acids 1–461; CBP2, amino acids 461–721; and CBP3, amino acids 721–1100) as bait to address this question. The CBP1, CBP2 and CBP3 proteins were immobilized on microtiter plates and incubated with the library, BSA served as an unspecific control. After three rounds of selection, the CBP2 protein, containing the major

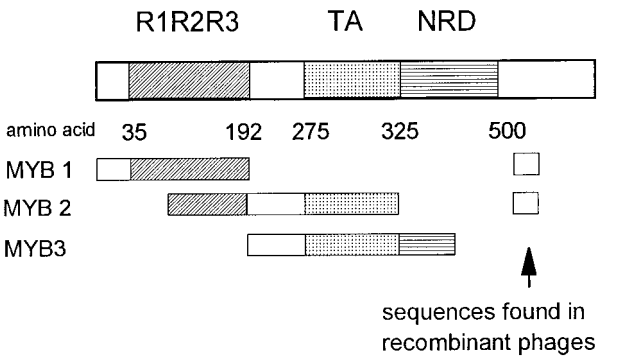

Fig. 3. Schematic view of the Myb protein and the bait proteins utilized in the experiment for the detection of intramolecular interactions. The truncated Myb1 protein contains the whole DNA binding domain (R1, R2 and R3), Myb2 consists of R2 and R3, the region between the DNA binding and the transactivating domain and the transactivating domain, Myb3 lacks the DNA binding domain and in addition to the transactivating domain, it spans a major part of the negative regulatory domain.

part of the CREB binding domain, had selected over 150 000 clones, while the other proteins did not give rise to clone numbers higher than background. The results are summarized in Table 1. Sequencing of 25 of the clones selected by the CBP2 protein revealed that they all shared a section of the c-myb gene encoding amino acids 312–413 of the protein; the minimal overlap of these sequences consists of positions 317–341. This region is located at the C-terminal end of the transactivating domain of Myb and N-terminal of the leucine rich region of the protein. The location of the sequence is depicted in Fig. 2.

3.4. Intramolecular interaction between the N-terminus and the C-terminus of Myb

The mini-library selection approach also provides a useful tool for studying intramolecular protein-protein interaction. In vivo studies have previously indicated [14] that the N-terminal DNA binding domain of the Myb protein interacts with

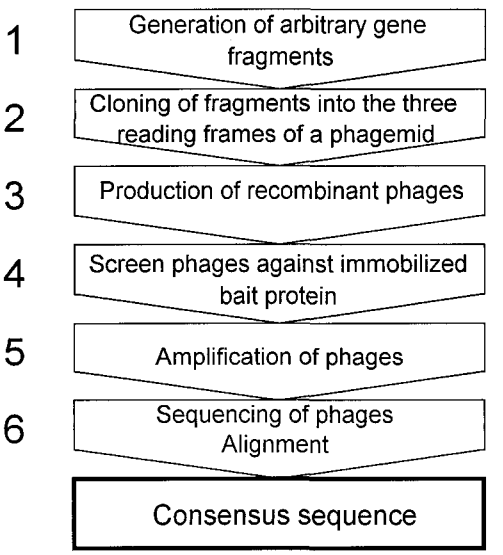

Fig. 4. Strategy of the phage display technique. The consensus sequence can be inferred from the alignment of sequences determined after the selection procedure.

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226 TTDGQQILVPSNQVVVQAAS 244 CREB.HS
153 TSDGQQILVPSNQVVVQTAS 172 TR36.HS
115 TSDGQQILVPSNQVVVQTAS 135 ATF1.HS
317 ELKGQQAL PT QNH  TAN 333 CMYB.MM

    GQQXLXPS
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Fig. 5. Comparison of the amino acid sequences in single letter code of three members of the human multigene family CREB (CREB, TR36 and ATF1) with the amino acid consensus sequence of the murine Myb protein selected by the CBP2 protein. The relative locations of the sequences within the proteins are indicated by numbers. Abbreviations: HS: *Homo sapiens*; MM: *Mus musculus*.

the C-terminus. In order to investigate whether our system can detect this intramolecular interaction, we expressed three truncated N-terminal Myb proteins and used them as bait for phage display selection. The outline of the experiment is shown in Fig. 3. The proteins Myb1 (N-terminal region and the full DNA binding domain (amino acids 1–93)), Myb2 (repeats 2 and 3 of the DNA binding domain and the transactivating domain (amino acids 90–326)) and Myb3 (transactivating domain and part of the negative regulatory domain (amino acids 193–414)) were expressed as His_6-tagged proteins and purified using Ni^{2+}-NTA affinity chromatography. The selection procedure was carried out as above with the modification that the Myb proteins were immobilized on Ni^{2+}-NTA-*HisSorb* strips. In this experiment, significant selection of recombinant phages was accomplished by the Myb1 and Myb2 bait proteins, suggesting that repeat 2 and repeat 3 of the DNA binding domain are involved in the interaction. Alignment of the sequence data established that the common feature of all phages bound by this protein was the expression of amino acids 541–567 of Myb.

4. Discussion

We have studied the intra- and intermolecular interactions of the c-myb protein using a phage display system. In general, our experimental approach (depicted in Fig. 4) can be divided into six steps. We have used the phage display method to examine in detail the interaction of the Myb protein and the CREB binding domain of the CBP cofactor. This interaction had been mapped previously by in vivo and in vitro experiments [9] to the CREB binding domain of CBP and amino acid positions 191–401 of the Myb protein. Our assay restricts this interaction to the amino acids 317–342. Since the CREB binding domain of CBP binds the CREB protein and related proteins such as ATF1 or TR36, it is not surprising that a motif shared by these proteins is also present in the 317–342 sequence detected by our screening procedure (cf. Fig. 5). Furthermore, it is important to note that in v-myb positions 321–330 are necessary for transactivation [15].

The phage display system is very sensitive and is able to detect intramolecular protein-protein interactions. We could confirm the data produced by Dash et al. [14], who proposed that the negative regulatory domain of Myb 'folds back' to bind to the amino-terminal DNA binding domain and thus regulates the protein's ability to transactivate. With a two-hybrid assay the authors mapped the region amino acids 506–562 (termed EVES domain) as the interacting partner of the Myb DNA binding domain. Again our phage display technique yields equivalent results: only bait containing the Myb DNA binding domain selected phages carrying coding sequences of the positions 541–567 within the EVES domain.

Phage display is a promising system for the detection of interaction partners with a variety of advantages:

- by means of several selection and amplification cycles, even low affinity interaction partners can be detected;
- unlike in an in vivo system, a stringent control of the experimental parameters is possible;
- under in vitro conditions, the structure of a discrete domain is decoupled from its biological function;
- the fusion of a protein to the phage g3p envelope protein increases its solubility and prevents protease degradation;
- since phage display uses exclusively microbiological techniques, its implementation is simple, less costly and less time demanding compared to eukaryotic in vivo systems.

Although the method is potent, fast and easy to handle, one should consider its limitations. One should keep in mind that the proteins displayed on the phage surfaces are more or less linear epitopes, which are not processed, i.e. do not carry phosphate or glycosylated modifications. The phage display system selects domains with strong binding affinity; however, protein regions with low in vitro affinity will not be bound although they might exhibit strong effects in vivo. It is obvious that a domain consisting of several weakly interacting regions contributing synergistically to an in vivo active motif might therefore not be detected by our approach. This was the case when we tried to perform the above experiment in reverse, i.e. using the respective Myb protein as a bait for the screening with a CBP library. Presumably, the full length 28 kDa CREB binding domain of CBP consists of several low affinity regions which act cooperatively in the binding process. Furthermore, the CREB binding domain is too large to be expressed as a g3p fusion protein [13]. Therefore, the clones selected by the Myb protein contained sequences which were randomly distributed over the whole CBP gene (data not shown). In conclusion, the expression of a mini-library in a phage display system is a straightforward, sensitive and fast approach for the mapping of protein-protein interactions.

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