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NMR Structure of the hRap1 Myb Motif Reveals a Canonical Three-helix Bundle Lacking the Positive Surface Charge Typical of Myb DNA-Binding Domains

Shingo Hanaoka[1], Aritaka Nagadoi[1], Shoko Yoshimura[2] Saburo Aimoto[2], Bibo Li[3], Titia de Lange[3] and Yoshifumi Nishimura[1,4]*

[1]*Graduate School of Integrated Science, Yokohama City University, 1-7-29 Suehiro-cho Tsurumi-ku, Yokohama 230-0045 Japan*

[2]*The Protein Research Institute Osaka University 3-1 Yamadaoka, Suita 565-0871, Japan*

[3]*The Rockefeller University New York, NY 10021, USA*

[4]*Genomic Sciences Center RIKEN Yokohama Institute 1-7-22 Suehiro-cho Tsurumi-ku, Yokohama 230-0045 Japan*

**Corresponding author*

Mammalian telomeres are composed of long tandem arrays of double-stranded telomeric TTAGGG repeats associated with the telomeric DNA-binding proteins, TRF1 and TRF2. TRF1 and TRF2 contain a similar C-terminal Myb domain that mediates sequence-specific binding to telomeric DNA. In the budding yeast, telomeric DNA is associated with scRap1p, which has a central DNA-binding domain that contains two structurally related Myb domains connected by a long linker, an N-terminal BRCT domain, and a C-terminal RCT domain. Recently, the human ortholog of scRap1p (hRap1) was identified and shown to contain a BRCT domain and an RCT domain similar to scRap1p. However, hRap1 contained only one recognizable Myb motif in the center of the protein. Furthermore, while scRap1p binds telomeric DNA directly, hRap1 has no DNA-binding ability. Instead, hRap1 is tethered to telomeres by TRF2. Here, we have determined the solution structure of the Myb domain of hRap1 by NMR. It contains three helices maintained by a hydrophobic core. The architecture of the hRap1 Myb domain is very close to that of each of the Myb domains from TRF1, scRap1p and c-Myb. However, the electrostatic potential surface of the hRap1 Myb domain is distinguished from that of the other Myb domains. Each of the minimal DNA-binding domains, containing one Myb domain in TRF1 and two Myb domains in scRap1p and c-Myb, exhibits a positively charged broad surface that contacts closely the negatively charged backbone of DNA. By contrast, the hRap1 Myb domain shows no distinct positive surface, explaining its lack of DNA-binding activity. The hRap1 Myb domain may be a member of a second class of Myb motifs that lacks DNA-binding activity but may interact instead with other proteins. Other possible members of this class are the c-Myb R1 Myb domain and the Myb domains of ADA2 and Adf1. Thus, while the folds of all Myb domains resemble each other closely, the function of each Myb domain depends on the amino acid residues that are located on the surface of each protein.

Keywords: Rap1; TRF1; Myb; telomeres; NMR

Introduction

Telomeres are the protein/DNA complexes that protect the ends of eukaryotic linear chromosomes from degradation and fusion. Mammalian telomeres are composed of long tandem arrays of the double-stranded telomeric repeat, TTAGGG, associated with the telomeric repeat-binding factors, TRF1 and TRF2.[1–5] Human TRF1 consists of 439 amino acid residues, containing three functional domains: an N-terminal acidic domain, a central TRF- homology (TRFH) domain that med-

Abbreviations used: BRCT, homology to the BRCA1 C-terminus; DQF-COSY, double quantum filtered correlated spectroscopy; 4D-SA, four-dimensional simulated annealing; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; RCT, homology to the Rap1 C terminus; TOCSY, total correlation spectroscopy; TRFH, TRF-homology domain; hRap1, human Rap1; scRap1, *Saccharomyces cerevisiae* Rap1.

E-mail address of the corresponding author: nisimura@tsurumi.yokohama-cu.ac.jp

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iates dimerization, and a C-terminal DNA-binding domain.[2,5,6] The DNA-binding domain of TRF1 contains a single Myb domain consisting of a 62 amino acid residue sequence that shows homology to each of three repeats of the c-Myb DNA-binding domain.[7,8]

The c-Myb protein is a transcriptional activator that binds to a consensus sequence of TAACNG[9–13] and regulates the proliferation of hematopoietic cells. The DNA-binding domain of c-Myb consists of three imperfect tandem repeats, R1, R2 and R3, each consisting of 52 amino acid residues. Each of c-Myb repeats has a very similar tertiary structure containing three helices[14–16] and the second and third helices form a helix-turn-helix (HTH) variant motif.[17–19] For the sequence-specific DNA binding of c-Myb, both R2 and R3 are essential and sufficient; however, R1 has no specific role in DNA-binding. In a DNA complex of c-Myb, R2 and R3 are closely packed in the major groove of DNA, recognizing a specific base sequence cooperatively.[15]

Like c-Myb, full-length dimeric TRF1 binds its telomeric TTAGGG repeat sites by engaging two Myb domains on DNA. In this binding mode, each of the Myb domains contacts a TAGGGTTAG site independently and with great spatial flexibility, and dimerization is required for stable complex formation *in vitro* and *in vivo*.[5,20,21] However, *in vitro* the isolated TRF1 Myb domain can bind specifically and with a significant affinity to telomeric DNA , recognizing a binding site centered on the sequence GGGTTA.[22] The solution structure of the Myb domain of TRF1 consists of three helices and the architecture of the three helices is very close to that of each of c-Myb repeats, containing the HTH variant motif.[23] However, the third helix is a little longer than the corresponding helix of each c-Myb repeat and the conformation of the turn of the HTH of TRF1 is slightly different from that of the corresponding turn of each c-Myb repeat. Based on the structure of TRF1, the N-terminal flexible arm of the Myb domain likely interacts with DNA from the minor groove in addition to the recognition of the HTH variant motif of TRF1 in the major groove of DNA like homeodomains.

The telomeres of the budding yeast *Saccharomyces cerevisiae* consist of 300-450 base-pairs of an irregularly repeated sequence motif $(TG)_{1\text{-}6}TG_{2\text{-}3}$ in contrast to the vertebrate regularly repeated sequence.[24–27] The budding yeast telomeric DNA is packaged by scRap1p, which contains an N-terminal BRCT (homology to the BRCA1 C terminus) domain, a central DNA-binding domain and a C-terminal RCT (homology to the Rap1p C terminus) domain. Although the DNA-binding domain of scRap1p shows no sequence similarity with other DNA-binding domains so far examined, it contains two subdomains, D1 and D2, connected by a long linker and each subdomain is structurally closely related to the c-Myb domain.[28]

Recently, the human ortholog of scRap1p was found by a yeast two-hybrid screen for proteins that interact with TRF2. Human Rap1 (hRap1) is distantly related to Rap1p from *S. cerevisiae* and *Klyveromyces lactis* in two protein interaction domains, the N-terminal BRCT domain and the C-terminal RCT domain. In addition, hRap1 contains a central Myb domain with sequence similarity to the D1 Myb domain of the budding yeast Rap1ps. However, hRap1 lacked the ability to bind to telomeric DNA *in vitro* and bound to telomeres *in vivo via* tethering by TRF2.[29] These results raised the possibility that the Myb domains of human and budding yeast may be functionally distinct. Here, we have determined the solution structure of the Myb domain of hRap1 by NMR and compared it with other Myb domain structures from TRF1, scRap1p and c-Myb. Although made up of the same fold, the hRap1 Myb domain has a distinct electrostatic potential surface from that of each Myb domain involved in DNA binding. Our findings raise the possibility that the hRap1 Myb domain, like R1 of c-Myb, does not interact with nucleic acid but rather associates with a protein partner. Alternatively, hRap1 may require an interacting factor that provides a basic surface allowing the hRap1 Myb domain to interact with DNA.

Structure determination

The Myb domain of hRap1, amino acid residues 132 to 190 (see Figure 1(b)), was chemically synthesized and subjected to the usual two-dimensional proton NMR experiments, double quantum filtered correlation spectroscopy (DQF-COSY),[30] nuclear Overhauser effect spectroscopy (NOESY)[31] and total correlation spectroscopy (TOCSY),[32] as reported.[14,16,23] Sequential and short-range nuclear Overhauser effect (NOE) connectivities are shown in Figure 2. These NOE patterns clearly indicate that the Myb domain of hRap1 contains three helical regions as found in R1, R2 and R3 of c-Myb and the TRF1 Myb domain.[14,16,23]

To determine the three-dimensional structure of the hRap1 Myb domain, we have obtained a set of 856 distance constraints; 260 intra-residue, 395 short-range and 194 long-range from NOESY spectra, and seven hydrogen bonds from a hydrogen-deuterium exchange experiment; and 39 angle constraints from a DQF-COSY spectrum. With these constraints, we have carried out distance geometry calculations using X-PLOR.[33]

The 25 calculated structures with amino acid residues that form a hydrophobic core are shown superimposed in stereo in Figure 3(a) and the lowest-energy structure is shown in Figure 3(b). None of the calculated structures showed violations greater than 0.3 Å for the distance constraints or 3° for the dihedral restraints. The backbone conformations are well defined in our calculations except the N-terminal four residues and C-terminal three residues. The overall rmsd values between the 25 individual structures and the mean coordinates are 0.61 Å for the backbone atoms and 1.02 Å for all heavy atoms, excluding the N and C

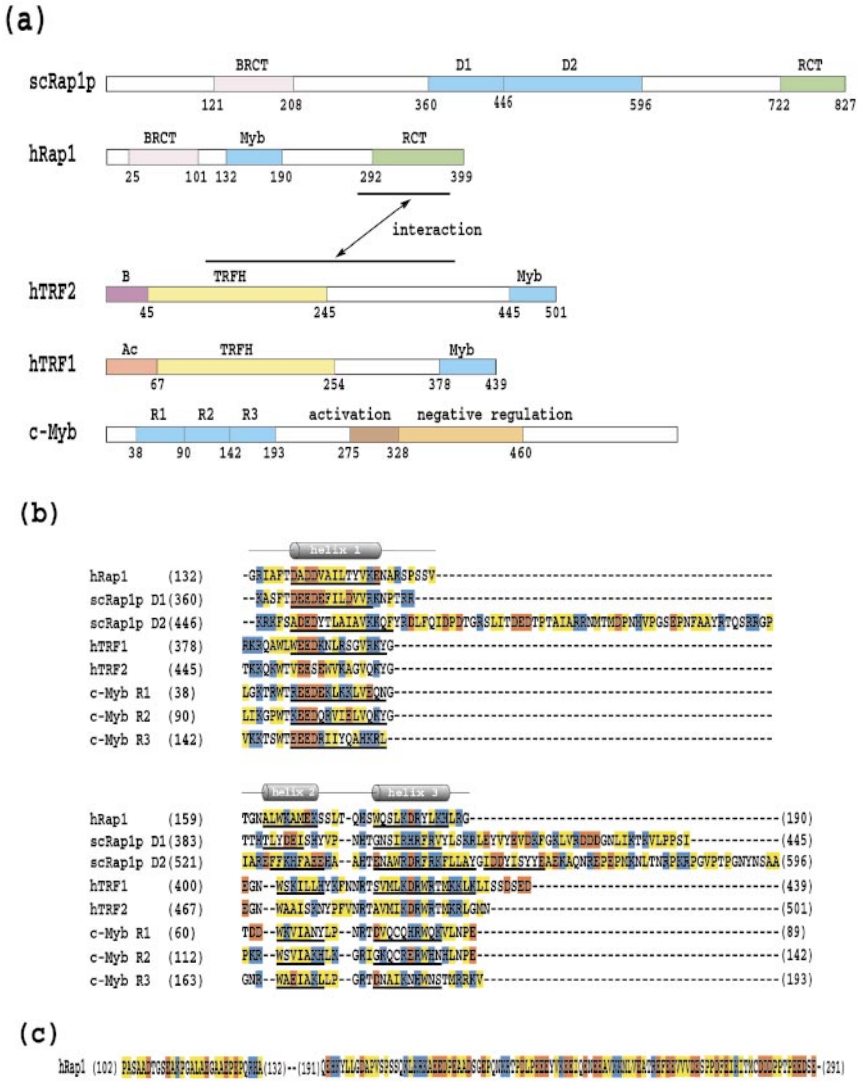

Figure 1. Domain structures and amino acid sequences of hRap1, scRap1p, TRF2, TRF1 and c-Myb. (a) Domain structures of scRap1p, hRap1, human TRF2, human TRF1 and c-Myb. BRCT, homology to the BRCA1 C terminus; Myb, Myb domain; D1, the first subdomain of the DNA-binding domain from scRap1p; D2, the second subdomain; R1, R2 and R3, the first, second and third repeats of the c-Myb DNA-binding domain, respectively; RCT, homology to the Rap1 C terminus; B, basic region; Ac, acidic domain; TRFH, TRF homology domain; activation, transcription activation domain; negative regulation, transcription negative regulation domain. (b) Sequence alignment of Myb domains of hRap1, D1 and D2 of scRap1p, hTRF1, hTRF2 and R1, R2, and R3 of mouse c-Myb. Yellow-colored residues are hydrophobic, red are acidic and blue are basic. The helical regions of the hRap1 Myb domain are shown at the top as cylinders and the helical regions of each domain are underlined in each sequence. (c) The amino acid sequence of hRap1 outside the Myb domain.

termini. These and other relevant statistics are summarized in Table 1. The Ramachandran plot of the 25 structures shows that, of non-glycine/proline residues, 70.4% residues are in the most favored regions and 25.4% residues are in the additional allowed regions, whilst 4.2% residues are in generously allowed regions by using the program PROCHECK.[34]

The structure consists of three helical regions: helix 1 (Asp138-Glu150), helix 2 (Ala162-Lys169), and helix 3 (Trp177-His187). The three helices are maintained by the hydrophobic core formed by residues Phe136, Ile144, Val147, Val158, Leu163, Trp164, Met167, Leu180, and Tyr184 as shown in Figure 3. The long loop between helices 1 and 2 forms a helical structure stabilized by Val158. Helices 2 and 3 form an HTH variant motif containing a three amino acid longer turn than the corresponding turn in the prototypic HTH proteins.[17–19]

Comparison with the Myb domains of c-Myb, TRF1 and scRap1p

Figure 4 shows the structure of the hRap1 Myb domain obtained here, with the structures of the TRF1 Myb domain,[23] and R1, R2 and R3 of

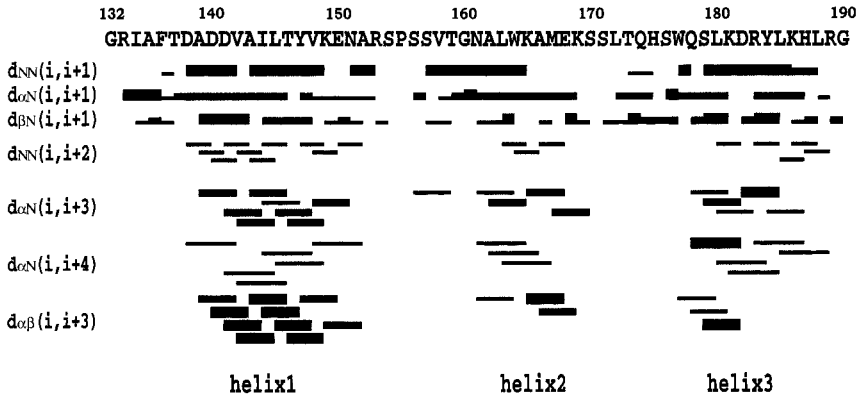

Figure 2. NOE connectivities of the hRap1 Myb domain. Boxed and gray regions are helical regions.

c-Myb.[16] The backbone architecture of the three helices is similar in the hRap1 and TRF1 Myb domains, three repeats of c-Myb and two subdomains of scRap1p. The rmsd values between the backbone atoms in the refined average structure of the hRap1 Myb domain, amino acid residues 138-150, 162-169, and 177-187, and the corresponding atoms of the TRF1 Myb domain, R1, R2 and R3 of c-Myb and D1 and D2 of scRap1p are 2.98, 2.63, 2.67, 2.70, 1.45 and 2.69 Å, respectively. Thus, the backbone architecture of the hRap1 Myb domain is most similar to that of D1 of scRap1p, corresponding that the amino acid sequence of the hRap1 Myb domain is most closely related to that of D1 of scRap1p.

In the hRap1 Myb domain, the length of the turn between the first and second helices consists of 11 amino acid residues, which is significantly longer than that of the corresponding turn in each of c-Myb repeats and the TRF1 Myb domain, consisting of three to four amino acid residues. In this sense, the hRap1 Myb is similar to the D1 domain of scRap1p, which has a nine amino acid residue turn. The D2 Myb domain of scRap1p has an atypical long flexible loop consisting of 54 amino acid residues between the first and second helices. Finally, the hRap1 Myb domain is similar to the D1 domain of scRap1p in its hydrophobic core, which contains phenylalanine and tyrosine residues in place of the three conserved tryptophan residues that hold together the three helices of each c-Myb repeat.

Table 1. Structural statistics for 25 hRap1 NMR structures

Distance restraints	
Intra-residue ($i - j = 0$)	260
Medium-range ($\|i - j\| < 5$)	395
Long-range ($\|i - j\| \geqslant 5$)	194
Hydrogen bonds	7
Total	856
Dihedral angle restraints	39
Statistic for structure calculations	⟨SA⟩
rmsd from experimental restraints	
NOE (Å)	0.0105 ± 0.0016
Dihedrals (deg.)	0.196 ± 0.132
rmsd from ideal restraints	
Bonds (Å)	0.0030 ± 0.0002
Angles (deg.)	0.537 ± 0.017
Impropers (deg.)	0.362 ± 0.007
rmsd of atomic coordinates (Å)	
Residues 136-187	
Backbone	0.61 ± 0.09
All heavy atoms	1.02 ± 0.08
Residues 138-150, 162-169, 177-187	
Backbone	0.39 ± 0.11
All heavy atoms	0.75 ± 0.17
PROCHEK Ramachandran plot statistics (%)	
Residues in most favoured regions	70.4
Residues in additional allowed regions	25.4
Residues in generously allowed regions	4.2
Residues in disallowed regions	0.0

Comparison of electrostatic surfaces of Myb domains and the relationship with the DNA-binding abilities of TRF1, c-Myb and scRap1p

Despite their similar fold, each Myb domain has a characteristic electrostatic potential surface that contributes to its function. Figure 4(a) shows that the TRF1 Myb domain has an overall strong positive surface that allows it to bind DNA even in the absence of dimerization.[20,22] On the basis of the structure of the TRF1 Myb domain and information on its DNA recognition sequence, we could make a reasonable model structure of the complex of TRF1 and DNA.[23] In the complex, the flexible N-terminal arm binds DNA in the minor groove in addition to the recognition of HTH variant motif in the major groove of DNA. This binding is reminiscent of the binding mode of homeodomains. Both antenapedia and engrailed homeodomains have an overall positive surface around each protein, like the TRF1 Myb domain, and, by using only one fold, each homeodomain could bind to DNA specifically.[35]

Figure 4(b) shows that both R2 and R3 of c-Myb have positive surfaces around their HTH motifs. As shown in Figure 4(c), both positive surfaces of R2 and R3 are required for the sequence-specific DNA binding in a cooperative manner. Figure 4(b) shows that the reverse side against HTH motif of each of R2 and R3 does not have a positive surface, so upon binding to DNA both positive surfaces of R2 and R3 should interact cooperatively with DNA. This is also supported by our NMR relax-

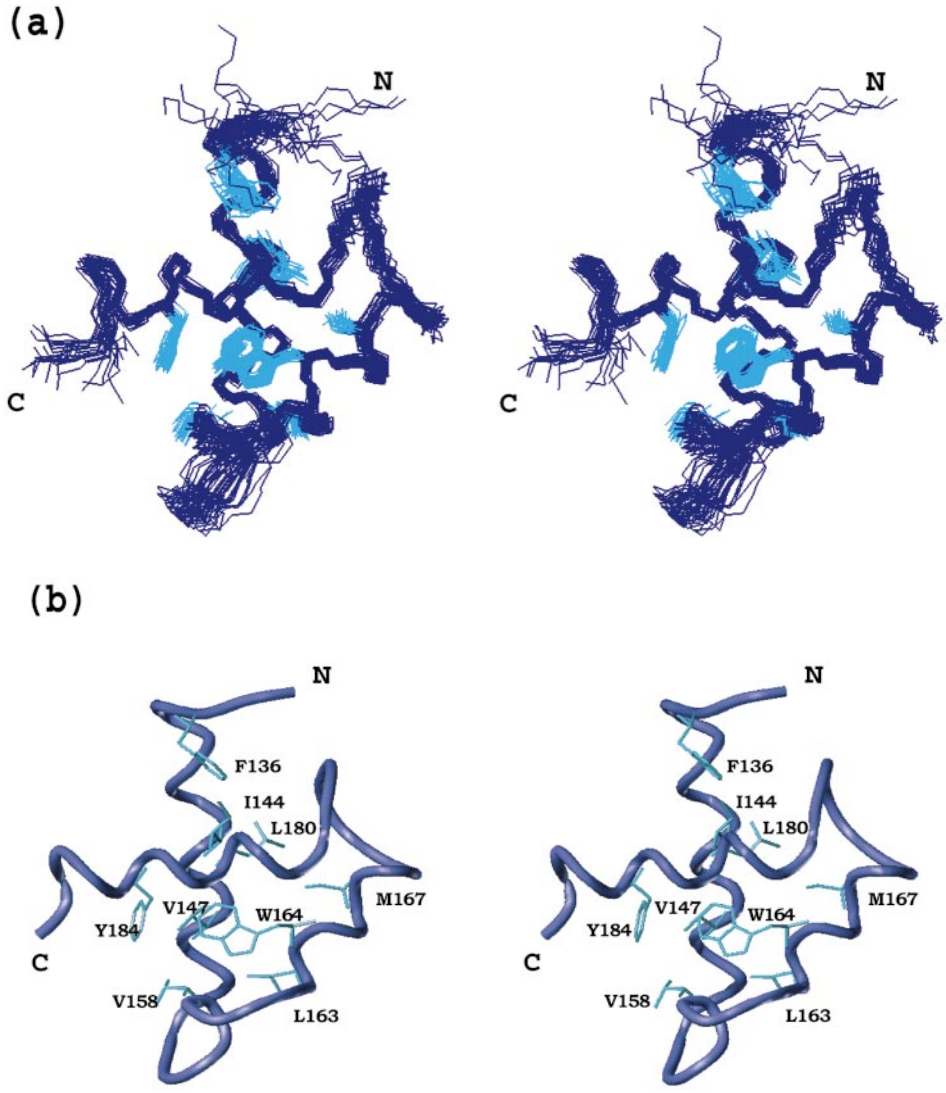

Figure 3. Stereoviews of the Myb domain structure of hRap1. (a) A stereoview of the 25 superimposed structures with amino acid residues that form a hydrophobic core of the Myb domain of hRap1. Cyan-colored residues form a hydrophobic core, maintaining the architecture of the three helices. (b) A stereoview of the lowest-energy structure in the 25 structures of the Myb domain of hRap1. Amino acid residues that form a hydrophobic core are indicated.

ation experiments of the minimal DNA-binding domain of c-Myb, the R2R3 fragment.[36,37] In the DNA-unbound state of the domain, the linker between R2 and R3 takes a flexible conformation, so each of R2 and R3 fluctuates independently on a nanosecond timescale.[36] Upon the complex formation with a specific sequence, the fluctuation of the linker of R2 and R3 is suppressed by the interaction with the DNA backbone, and R2 and R3 become fixed in the major groove. Furthermore, the conformational fluctuations between the recognition surfaces of R2R3 and DNA are slowed to the millisecond timescale.[37] It is likely that, without DNA, the positive surfaces of R2 and R3 are repulsive so that R2 and R3 move independently each other, while in the DNA complex both positive surfaces wrap around the negative surface of the DNA backbone.

In contrast to R2 and R3, R1 of c-Myb has no distinct positive area. This is of interest since, like the hRap1 Myb domain, R1 may not interact with DNA. R1 is not required for the sequence-specific binding of c-Myb, which can be executed fully by R2/R3. However, a deletion of R1 enhances the oncogenic potential of c-Myb and R1 is essential for the normal regulation of cell division and proliferation.[38] A likely explanation for these findings is that R1 interacts with another (unidentified) protein, rather than with DNA.

The three helical bundles that make up the D1 and D2 Myb domains of scRap1p lack distinct positive areas. However, as shown in Figure 4(d), the linker between D1 and D2 cooperates with the loop between the first and second helices of D2 and the flexible C-terminal tail after D2 to form strong positive surfaces in the DNA complex.

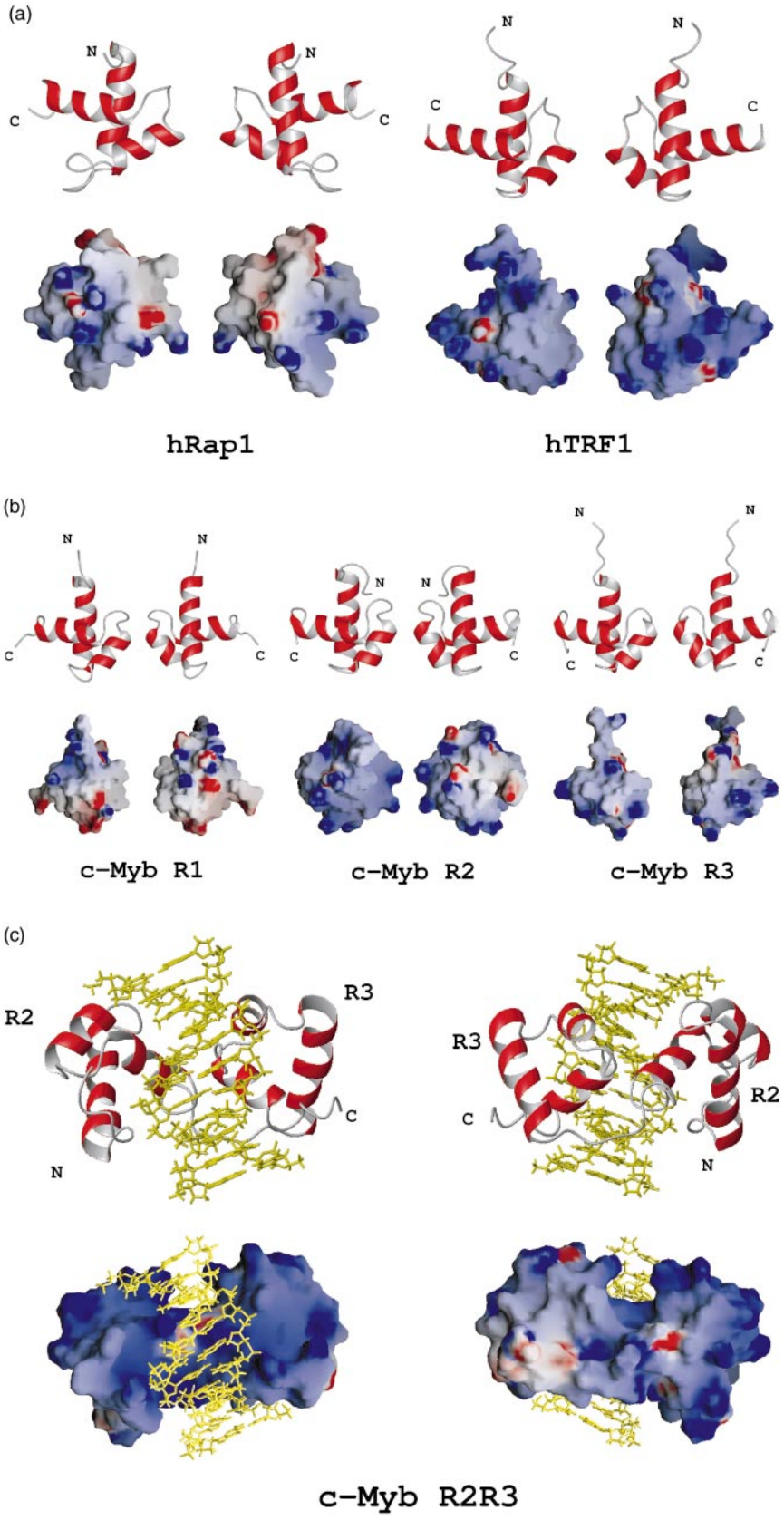

Figure 4 (*legend opposite*)

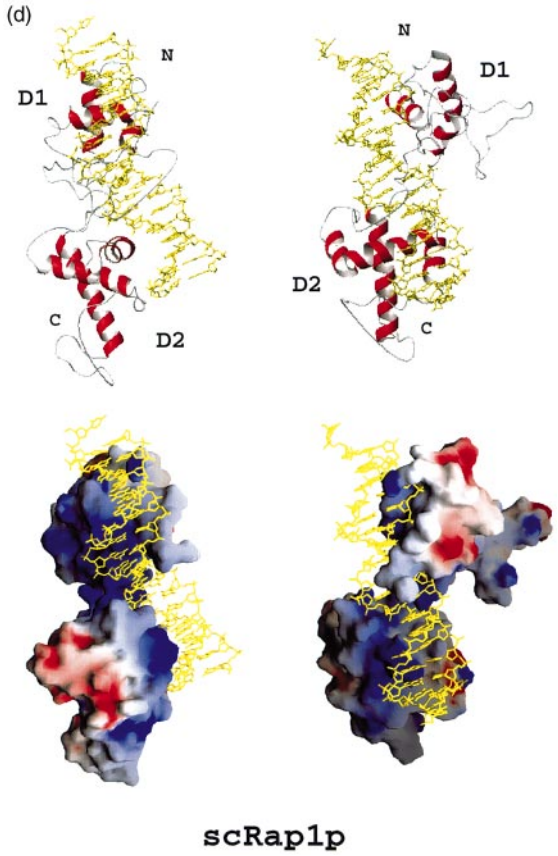

Figure 4. The three-dimensional structures and electrostatic potential surfaces of the Myb domains in the free and DNA-bound forms. (a) The hRap1 and hTRF1 Myb domains, (b) R1, R2 and R3 of c-Myb, (c) the R2R3-DNA complex of c-Myb, and (d) the DNA-binding domain of scRap1p bound to DNA are shown. In each of the Myb domains in free forms the left and right panels correspond to the HTH side and the reverse side, respectively. In the DNA complex of c-Myb the left and right panels correspond to views from a DNA side (shown as a wire model) and the reverse side, respectively. In the DNA complex of scRap1p the left corresponds to a view of D1 from a DNA side (shown as a wire model) and the right is a view of D2 from a DNA side. In each panel the upper shows the protein structure with a ribbon model and the lower shows the corresponding electrostatic potential surface. Blue and red-colored regions correspond to positive and negative regions, respectively.

Overall, this positive surface forms an extensive and broad DNA-binding interface. It is likely that in the DNA-unbound state both D1 and D2 have a fold similar to the Myb domain; however, the linker between D1 and D2, the long loop in D2 and the C-terminal tail are likely to be disordered in the absence of DNA and fluctuate randomly. Thus, in the absence of DNA, electrostatic repulsion is likely to interfere with the formation of an extensive positive surface. This view is consistent with the finding that D1 alone has no DNA-binding ability and that deletion of amino acid residues, 583-596 just beyond D2 causes loss of DNA-binding activity.[39]

The hRap1 Myb domain lacks a distinct positive surface itself, as shown in Figure 4(a). Furthermore, unlike the area around scRap1p D1 and D2, the regions surrounding the hRap1 Myb domain lack significant numbers of positive amino acid residues, as shown in Figure 1(c). We suggest that the lack of extensive positive surface is responsible for the lack of DNA-binding activity of hRap1 *in vitro*. It is possible that the hRap1 Myb domain could be involved in DNA by interaction with another protein that provides this positive surface. An interesting candidate might be its interacting partner TRF2, which has a very basic N terminus. Alternatively, hRap1 may not be a DNA-binding protein at all, and its Myb domain could be involved in protein-protein interaction. Precedent for this novel role for a Myb domain comes from the work on c-Myb R1 and from recent works on Adf1[40] and on ADA2.[41] In each of these proteins, a Myb-like HTH motif is implicated in protein binding. However, if the Myb domain of hRap1 is involved in protein-protein interaction, this would constitute the first known example of a functional switch for a conserved domain in orthologous genes. Clarification

of this issue will await identification of hRap1 binding partners.

Materials and Methods

Sample preparation

The peptide fragment of hRap1 Myb domain was chemically synthesized and purified as described.[14,16,23] The lyophilized sample was dissolved in 100 mM potassium phosphate buffer (pH 5.5), 1 mM NaN_3 and the sample concentration was 1.0 mM.

NMR spectroscopy

NMR spectra were recorded at 600 MHz or 500 MHz on a Bruker DMX-600 or AMX2-500 spectrometer. The temperature during data acquisition was set to 300 K. Quadrature detection was made by the TPPI method. For water signal suppression, weak presaturation was used. DQF-COSY spectra,[30] NOESY spectra[31] with mixing times of 50, 100 and 150 ms; a TOCSY spectrum[32] with a mixing time of 100 ms were recorded, and relaxation-compensated DIPSI-2 mixing schemes with z filtration[42] were used in the pulse sequence of the TOCSY experiments. In 90 % H_2O/10 % ^2H_2O (v/v) and in 100 % ^2H_2O, 512 $t1$ increments (zero-filled to 1024 data points) each of 2048 real data points was recorded with a spectral width of 8390 Hz. In addition, a hydrogen-deuterium exchange experiment has been done by a DQF-COSY spectrum[30] with 512 $t1$ increments each of 8192 real data points (zero-filled to 8192 data points) two days after the dissolving the sample into ^2H_2O. Sequence-specific assignments could be completed from Gly132 to Gly190.

Structure calculations

Interproton distance constraints were derived from the cross-peak intensities of the NOESY spectra with mixing times of 50, 100 and 150 ms, using assumptions similar to previous calculations.[14,16,23] From the NOE intensities, the distances between protons were classified into three ranges, 1.8 to 3.0, 1.8 to 4.0, and 1.8 to 5.0 Å, corresponding to strong, medium, and weak NOEs, respectively. In addition, the ϕ angles were restrained by estimating the $^3J_{HN\alpha}$ coupling constants from F2 high-resolution DQF-COSY spectrum with corrections for deviated values due to broadened signals. The restrained angle ranges as follows: $-90^\circ < \phi < -40^\circ$ for $^3J_{HN\alpha} < 6.5$ Hz and $-160^\circ < \phi < -80^\circ$ for $^3J_{HN\alpha} > 8.5$ Hz.

Protein Data Bank accession number

The coordinates of the 25 calculated structures have been deposited in the Protein Data Bank. The PDB ID code is 1FEX. The NMR data have been deposited in BMRB with accession number 4639.

Acknowledgments

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