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Subunit interaction in the CCAAT-binding heteromeric complex is mediated by a very short α-helix in HAP2

(mutagenesis/protein–protein interaction/transcription complex)

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ABSTRACT **We dissected the domain of HAP2 that mediates subunit association in the heteromeric CCAAT-binding complex, first by genetic mutational analysis and then by structural studies. The mutational data suggest that a very short region in HAP2 mediates protein–protein association and that the structure of this domain is likely to be an α-helix. The CD analyses of a 15-residue synthetic oligopeptide covering this region confirm this surmise. The oligopeptide indeed formed an unusually thermal stable α-helix in aqueous solution. Eight amino acids that lie along one face of this helix, including three arginines, are found to be critical for protein–protein association. The partner that interacts with this helical motif is likely to be another subunit in the HAP complex, since the CCAAT-binding factor is shown to contain one molecule of HAP2. Our results suggest that very short regions in proteins can encode precise structures and mediate stable and specific protein–protein recognition and interactions.**

The specificity and stability of protein–protein interactions are critical determinants in the assembly of macromolecular complexes consisting of protein subunits. One well-studied example of these complexes involves the transcription factors Fos and Jun (for review, see refs. 1 and 2). Jun–Jun homodimers and Fos–Jun heterodimers will form readily and are active in cells, whereas Fos–Fos dimers do not form (3–5). Inspection of a family of transcription factors with DNA-binding domains like those of Fos and Jun led to the leucine zipper hypothesis (6).

A heteromeric transcriptional activator that was identified early on is the yeast (*Saccharomyces cerevisiae*) CCAAT-box binding factor HAP2/3/4 (7–9). Both HAP2 and HAP3 were present in a gel-shift complex with a CCAAT box-containing probe (9), and HAP2 and HAP3 remained stably associated in the absence of DNA (8). The DNA-binding function of this complex as it is found in yeast consists of the HAP2 and HAP3 subunits (10), and the transcriptional activation domain is provided by a third subunit, HAP4 (7). HAP2 and HAP3 are functionally and structurally conserved in other eukaryotes, including mammals (10–15).

The 265-aa HAP2 subunit was pared down to a 60-aa core which possessed all of the functions of the intact molecule (14). This core is highly conserved in *Schizosaccharomyces pombe*, mouse, rat, and human (15). Further analysis of the core defined two adjacent subdomains, a 20- to 40-aa region required for subunit assembly of the complex and an adjacent 21-aa domain necessary for binding to DNA. The DNA-binding domain of HAP2 functions in concert with a DNA-binding region in HAP3 (10).

The subunit-assembly domain (SAD) of HAP2 was sufficient for assembly of the complex in the absence of the DNA-binding domain, because a LexA chimera containing the SAD would form an active HAP2/3/4 complex at a LexA site *in vivo* (10, 14).

Here we describe the dissection of the SAD of HAP2 by genetic mutational and biophysical structural analyses. Our findings delimit the region and pinpoint which residues in HAP2 are critical for its function. The mutational data suggest that a very short region in HAP2 is likely to form an α-helix which mediates assembly. Our findings show that 8 aa along one face of this helix dictate the specificity and at least part of the stability of interaction between HAP2 and its interacting partner. Further, a 15-aa synthetic peptide covering this region showed a typical α-helical circular dichroism (CD) spectrum. We conclude that very short peptide regions can encode precise structures and mediate stable and specific protein–protein recognition and interactions in macromolecular complexes.

MATERIALS AND METHODS

Strains, Plasmids, and β-Galactosidase Assays. The *S. cerevisiae* strain BWG 1-7a (*MAT* a *leu2-3,112 his4-519 ade1-100 ura3-52*) or isogenic derivatives were used in all experiments described. HAP2 complementation was assayed by using strain JO1-1a (14). Vectors pRB1155 (16), pJO323 (14), and pLGΔ265UP1 (17); plasmid pXY3236 (10); and the assay for β-galactosidase activity have been described (10).

Saturation Mutagenesis of HAP2. Single-stranded DNA of plasmid pYX3236 was prepared and saturation mutagenesis on the HAP2 core region expanded from aa 154 to 182 was conducted as described (10).

Western Blotting. Extracts (2.5 μl) prepared from yeast cells carrying the wild-type or mutated *lexA–HAP2* plasmid were fractionated by SDS/PAGE and the separated proteins were transferred onto a nitrocellulose filter. The filter was incubated with guinea pig anti-LexA antiserum (gift from Jun Ma, Harvard University) and then with peroxidase-conjugated antibody (goat anti-guinea pig IgG; Sigma). The LexA–HAP2 proteins on the filter were visualized with x-ray film (ECL Western blot detection system; Amersham).

HAP2 Subunit Composition of the CCAAT-Binding Complex. *In vitro* transcription and translation (14) of full-length HAP2 and N- and C-truncated versions of HAP2 were carried out with pSP64:HAP2 and pSP64:HAP2ΔN154ΔC218 templates in rabbit reticulocyte lysates (Promega). Cotranslation of the two size variants was achieved by mixing two products of *in vitro* transcription reactions before programming the lysates. The resulting translation products were then subjected to DNA binding and gel shift analysis (14).

CD Analysis of Oligopeptide of HAP2 SAD. A 15-aa synthetic peptide covering the HAP2 subunit-association motif was chemically synthesized and HPLC purified. The peptide was dissolved at 0.5 mM in water. The stock peptide was

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Abbreviation: SAD, subunit-assembly domain.

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adjusted to 40 μM in 4 mM sodium phosphate/1 mM boric acid/200 mM NaCl, pH 7.0) and incubated overnight at 4°C before CD analysis (model 60DS spectrophotometer; Aviv Associates, Lakewood, NJ) at 4°C, 10°C, and at 10°C intervals up to 90°C. At each temperature, the sample was well equilibrated before the measurement was conducted.

RESULTS

Saturation Mutagenesis of the HAP2 SAD. A previous study delimited the HAP2 SAD to aa 154–197 (14). Since that work, the DNA sequences of HAP2 functional homologs from *Sch. pombe* (13), rat (18), mouse (19), and human (11) were shown to contain very strong similarity to HAP2 aa 162–182 (11, 19). The adjacent 11 aa on the C-terminal side are very divergent, and the following 21 aa, comprising the DNA-binding domain, are again very similar (10). This sequence comparison suggests that the SAD may lie within the 162–182 interval. Furthermore, replacement of aa 180–184 with completely dissimilar sequences did not disrupt HAP2 function (14). We thus entered these studies with the view that the SAD probably lay between aa 162 and aa 180.

Saturation mutagenesis of LexA–HAP2 residues 154–182 was carried out as described for the DNA-binding domain (10). We employed oligonucleotide pools spanning 11 aa that would cover the 154–182 interval. The DNA synthesis was calculated to generate mutations at a rate of one per molecule. The products of mutagenesis were transformed into a Δ*hap2* strain bearing a *lacZ* gene under control of the CCAAT box in UAS2UP1.

Mutants that resulted in white or pale blue colonies on plates with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside were selected for further analysis. We also chose for analysis some wild-type (blue) colonies. Mutations giving rise to stop codons were recovered among the white colonies. Stop-codon mutants would necessarily be defective regardless of whether they truncated the SAD because they would all remove the DNA-binding domain. Mutants generating stop codons at residues R175, Y176, R178, and K180 were transformed into a strain with the LexA-binding reporter so that the integrity of the SAD could be determined. By this assay, mutants generating stop codons at R175, Y176, and R178 could not assemble the HAP complex at the LexA site, whereas mutants with stop codons at residue 180 could (Table 1). This delimits the C terminus of the SAD to Y178 or A179 of HAP2 (Fig. 1).

Strikingly, missense mutations that gave rise to appreciable defects in activity were limited to aa 163–176 (Table 2).

Table 1. Determination of the C terminus of the HAP2 SAD by stop-codon mutations

LexA–HAP2	Growth on lactate plate	β-galactosidase, units: UAS2UP1 (CCAAT)	β-galactosidase, units: LexA operator
Wild type	++	108	35
Mutants			
R175Stop	−	2.3	0.3
Y176Stop	−	1.8	0.2
R178Stop	−	0.7	1.5
K180Stop	−	1.4	20.6

In vivo activity was determined by measuring β-galactosidase levels from pLGΔ265UP1 (CCAAT) and pRB1155 (LexA operator) in strain JO1-1a (Δ*hap2*). β-Galactosidase levels were obtained from cells grown in synthetic medium supplemented with 2% glucose. Complementation of the Δ*hap2* deletion in JO1-1a for growth on YEP lactate plates was also tested. Growth in lactate medium is indicated as full growth (++) or no growth (−). All stop-codon mutants fail to complement and fail to activate the CCAAT reporter because they are missing the HAP2 DNA-binding domain.

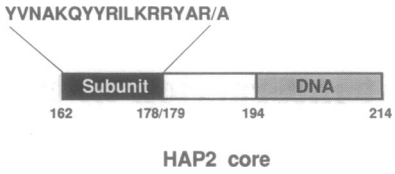

FIG. 1. Minimal motif of the SAD of HAP2. The HAP2 essential core is aa 162–214. Black box represents the SAD. Gray box represents the DNA-binding domain (10, 14).

Mutants that showed a defect at the CCAAT site were similarly defective at the LexA site, showing that these mutants are truly defective in subunit assembly. Further, the ability of the mutants to complement the Δ*hap2* strain for growth on lactate plates also strictly correlated with the reporter assays.

To be certain that these mutants did not owe their defects to destabilization of the protein, Western blot analysis was carried out with the LexA antiserum. All of the defective mutants tested contained a level of the LexA–HAP2 core protein that was comparable to the wild-type level (Fig. 2).

Critical Residues in the HAP2 SAD Domain. Mutations that caused a severe defect in subunit assembly were limited to 8 aa within the 163–175 interval which were regularly 3–4 aa apart: V163, N164, Q167, Y168, R170, I171, R174, and R175 (Table 2). Mutations with less effect on assembly were found at A177 and R178. Mutations that did not affect HAP2 function lay at Y162, K166, K173, Y176, and K180 and also included some conservative changes at the important residues Y168, I171, R174, and R178.

To verify biochemically that these mutants were defective, a gel mobility-shift assay was performed with extracts from strains expressing representative severe mutants and neutral mutants. The severe mutations abolished the HAP2/3 gel shift complex with the CCAAT probe, whereas the neutral mutations did not (Fig. 3).

The severe *hap2* mutations fell into four adjacent pairs of residues within the 162–175 interval, positions 163 and 164, 167 and 168, 170 and 171, and 174 and 175 (Fig. 4). Within each pair, the N-terminal residue appeared to be the more critical, because the more severe mutations were found at that position. However, the small sample size of only one or two mutations at the C-terminal residue of each pair precludes a strong conclusion on this issue. Importantly, the regular 3- to 4-aa spacing of these pairs of residues indicated that they would all lie along one side of an α-helix—i.e., every 300°–400° on the helical cylinder (Fig. 5). Interestingly, mutations affecting SAD function were found in *every* residue along the same side of the helical cylinder. All positions bearing only neutral mutations lay along the other side of the helix (see *Discussion*).

CD Analysis of the HAP2 SAD. Because the mutational analysis gave a strong indication that the active structure of the HAP2 SAD was an α-helical motif, a 15-mer peptide corresponding to residues 162–176 of HAP2 was chemically synthesized. Position 176 was deemed a likely terminus of an

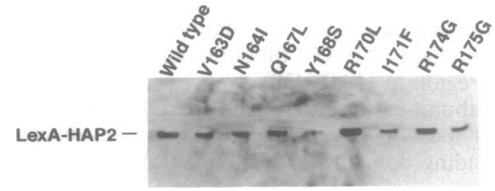

FIG. 2. Western blot assay of yeast extracts containing LexA–HAP2 and its mutants. Wild-type LexA–HAP2 protein is at left (wild type), and other lanes show mutant proteins as indicated.

Table 2. Site-directed mutagenesis of the protein–protein association domain of HAP2

LexA–HAP2 protein	Growth on lactate plate	β-Galactosidase, units UAS2UP1 (CCAAT)	β-Galactosidase, units LexA operator
Wild type	++	110	30
Mutants			
Y162C, Y168D	++	72.3	18.7
Y162F, N164I	−	4.3	0.1
V163D	−	5.4	0.5
N164I	−	9.6	0.2
N164L	−	10.7	0.4
N164K	+	38.7	5.2
N164Y	+	32.6	7.5
A165S, K166P	−	4.3	0.2
K166M	++	82.0	28.5
K166Q	++	89.7	19.8
Q167E	−	3.4	0.25
Q167L	−	3.7	0.3
Q167K	−	4.5	0.5
Y168S	±	9.4	0.75
Y168A	+	25.2	22.4
Y168N	+	31.6	17.2
Y168C	++	82.1	21.5
Y169F	++	85.0	25.6
R170P	−	3.4	0.4
R170L	−	6.0	0.45
R170G	−	9.8	0.3
I171F	±	12.2	4.4
I171T	+	45.7	6.3
I171V	++	94.3	26.5
K173N	++	92.8	21.4
L172W, R174M	−	4.7	0.2
R174G	−	6.2	0.3
R174W	±	11.3	0.4
R174S	±	8.7	0.4
R174K	++	77.6	25.5
R175G	−	8.1	0.6
R175Q	+	37.6	4.8
R175P	−	4.1	0.1
Y176P	++	97.6	22.3
Y176S	++	103.1	23.2
A177P	+	72.5	21.5
A177E, R178G	+	68.0	18.2
R178K	++	116	22.9
R178S	+	56.3	14.8
R178G	+	55.2	19.7
K180E	++	89.6	24.1

In vivo activity was determined by measuring β-galactosidase levels from reporters pLGΔ265UP1 (CCAAT) and pRB1155 (LexA operator) in strain JO1-1a. β-Galactosidase levels were obtained as described in Table 1. Growth in lactate medium is indicated as full, wild-type growth (++), intermediate growth (+), poor growth (±), or no growth (−).

α-helix because mutations of Y176 or A177 to proline did not disrupt function (see Table 2).

The CD spectra of the oligopeptide displayed two minimal intensities at 221 nm (n–Π* transition) and at 208 nm (Π–Π* transition), indicating a typical α-helical structure (Fig. 6*A*). The α-helical content of the oligopeptide is about 40% at 4°C as calculated at 208 nm (20). This wavelength was used because of the tyrosine content (4 out of 15) of the oligopeptide, which may have led to underestimation of the helical content if 221 nm had been used for the calculation. The structure is probably in an equilibrium between α-helix and random coil in aqueous solution. The SAD peptide of HAP2 is one of the shortest peptides shown to form an α-helix *in vitro*. Most stable α-helical structures of oligopeptides have

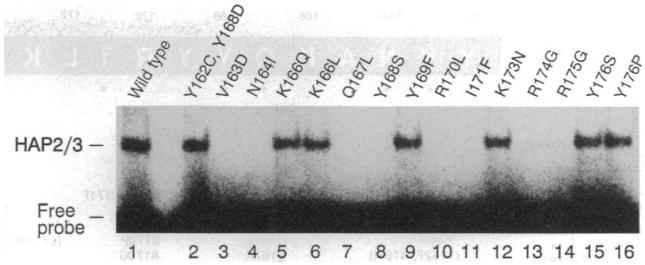

FIG. 3. Gel shift assay of yeast extracts containing wild-type and mutant LexA–HAP2 with a CCAAT-box probe. Lane 1, wild-type LexA–HAP2; lanes 3, 4, 7, 8, 10, 11, 13, and 14, LexA–HAP2 bearing the indicated defective mutations; lanes 2, 5, 6, 9, 12, 15, and 16, LexA–HAP2 bearing the indicated neutral mutations. Extracts of cells containing wild-type and neutral LexA–HAP2 were prepared from growing cells in lactate medium; extracts of cells containing defective mutations of LexA–HAP2 were prepared from cells in glucose synthetic medium. Both media give rise to a robust signal of HAP2/3 binding to a CCAAT-box probe (10).

a preference for alanines, with other residues at a defined spacing (21). Substitution of these alanines or changing their spacing drastically destabilizes their helical structures (22). Although the SAD peptide contains alanine residues, they are not sensitive to mutation (Table 2). Further, there is neither a defined spacing nor an ionic pair as defined by Marqusee *et al.* (23) in the SAD peptide.

The intensity of the minimum at 221 nm ($-[\theta]_{221}$) can be followed to measure the helical content as a function of temperature and thus the stability of the structure (21). We determined the CD spectra at various temperatures and plotted temperature versus $-[\theta]_{221}$ (Fig. 6*B*). The α-helix was still observed at temperatures up to 90°C but was slightly destabilized at higher temperatures. The intensity at 221 nm decreased about 15% at 90°C, with a major transition point at 60°C. Thus α-helix, once formed, has considerable thermal stability.

The HAP Complex Contains One Molecule of HAP2. Because α-helices in zipper proteins can mediate homodimer formation, we investigated whether the HAP2 SAD might promote dimerization of HAP2 in the HAP complex. Full-length HAP2 (bearing 265 aa) and a functional deletion version bearing the entire core (ΔN154ΔC218) (14) were cotranslated in a rabbit reticulocyte lysate *in vitro* and employed in gel shift assays. Previous experiments showed that these two HAP2 species gave rise to gel shift complexes of distinct mobility when mixed with a human fraction containing the HAP3 homolog CP1A and a CCAAT-box DNA probe (14). Two CCAAT complexes were observed by gel shift corresponding to each of the HAP2 variants, but a third complex intermediate in size between the two was not observed (Fig. 7, compare lane 4 with lanes 2 and 3). The same result was observed when HAP2 and its size variant were translated separately and then mixed with the CP1A fraction and CCAAT-box probe (data not shown). Thus, each of the *in vitro* translated HAP2 variants is capable of assembling into a HAP complex active for CCAAT binding, but a complex containing both of the variants does not form. The most reasonable conclusion from this experiment is that there is only one molecule of HAP2 per HAP complex. Thus, we surmise that the protein partner for the HAP2 SAD is a different subunit in the HAP complex—e.g., HAP3.

DISCUSSION

The CCAAT-binding heteromer HAP2/3 is conserved in eukaryotes ranging from yeast to mammals (15). The HAP2 subunit has been cloned from several divergent sources and found to bear a 60-aa region that is highly conserved (15).

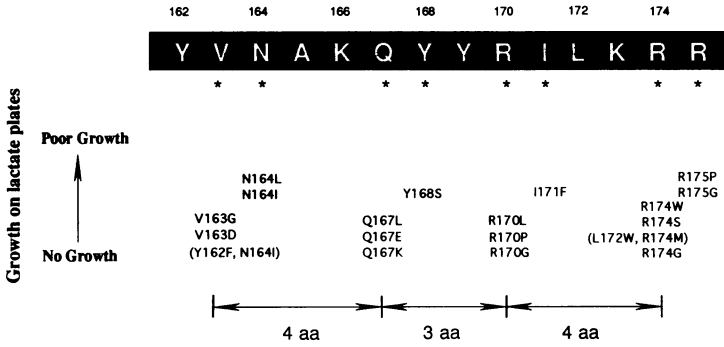

FIG. 4. Spectrum of mutations in the HAP2 SAD. Positions of strongly defective mutations are marked with stars. The spacing between the pairs of mutationally sensitive residues is indicated.

Within this region two domains have been defined, one for protein–protein interaction within the HAP complex (toward the N terminus) and another for DNA binding (toward the C terminus). The DNA-binding domain contains 21 aa and directly recognizes the CCAAT sequence (10). A spacer region between these two domains is divergent among the HAP2 sequences from various organisms. Further, the spacing between the two domains is variable (the *Sch. pombe* spacer contains one extra residue; ref. 13) and can be altered by a deletion/substitution resulting in a net removal of 3 aa (14).

A sequence comparison of the HAP2 SAD shows strong conservation at aa 162–182 (15). Residues 180–184 can be altered without any effect on function (14). In this study, we conducted a saturation mutagenesis of residues 154–182 of HAP2 in a chimera bearing a LexA DNA-binding domain at the N terminus, using pools of synthetic oligonucleotides made to produce one mutation on average per mutagenized gene. Eight residues which lay between aa 163 and 175 were mutationally sensitive in two *in vivo* assays. The first assay was activation of a reporter *in vivo* driven by the CCAAT sequence, a measure of HAP complex assembly and DNA-binding (17). The second assay was activation of a reporter driven by a LexA binding site, a measure solely of HAP complex assembly (24). All mutants isolated were defective in both assays, as well as complementation of a Δ*hap2* strain for growth on lactate. However, the LexA–HAP2 proteins produced by all of the mutants were stable as judged by Western blot analysis.

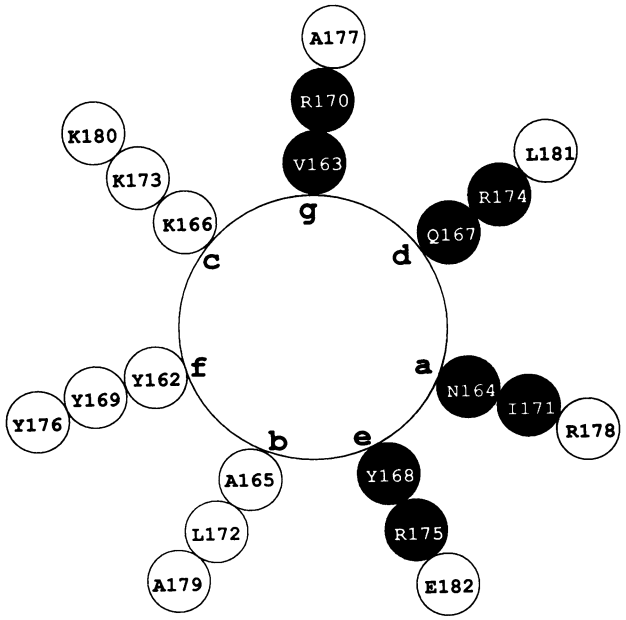

FIG. 5. Helical projection of SAD of HAP2. Functionally important amino acid residues are in black circles.

Inspection of the positions of the residues that were mutationally sensitive showed a regular 3- to 4-aa spacing that would place all 8 aa on the same side of an α-helix (Fig. 5). Residues that were not mutationally sensitive and gave rise solely to neutral mutations would all lie on the other side of the α-helix. Because of this pattern, we synthesized a peptide comprising residues 162–176 of the SAD and showed by CD spectra that it possessed α-helical character in solution. This structure was remarkably stable to increases in temperature up to 90°C.

In zipper interactions, such as Jun–Jun or Jun–Fos, strong interactions between residues of the *a* and *d* positions help cement the interaction, while interactions between residues of the *e* and *g* positions can influence the specificity of interaction—e.g., preventing interaction between Fos and itself due to electrostatic repulsion (4). By analogy and

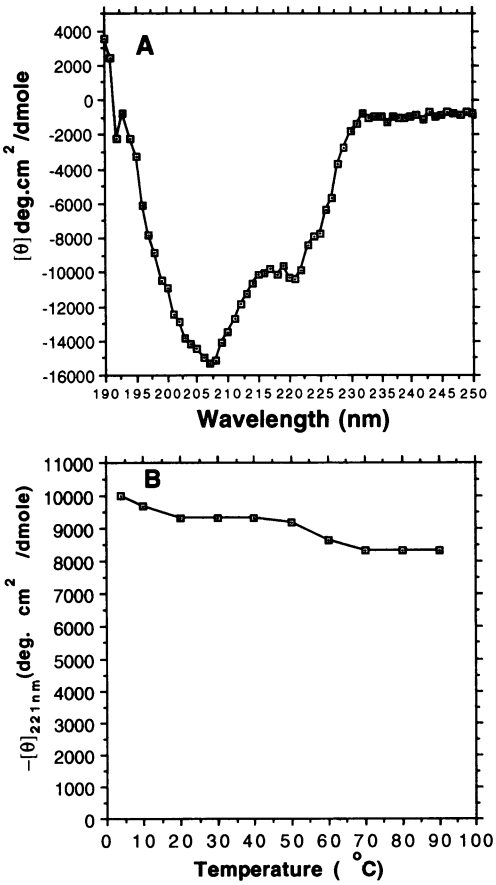

FIG. 6. CD studies of the 15-aa synthetic peptide spanning Y162–Y176 within the SAD. (*A*) CD spectrum of the peptide, with minima at 221 nm and 208 nm, indicates an α-helical structure with a calculated helical content about 40% (20). (*B*) Thermal stability of the peptide. Plot of temperature vs. ellipticity ($[\theta]_{221}$) is shown. The overall decrease in $[\theta]_{221}$ is about 15% from 4°C to 90°C.

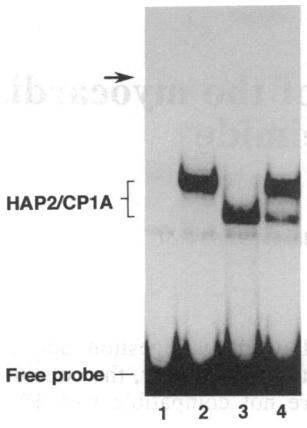

FIG. 7. HAP complex contains one molecule of HAP2. Gel shift assays were performed with *in vitro* translated HAP2 proteins and the mammalian HAP3 CP1A. Lane 1, CP1A alone as a control; lane 2, full-length HAP2 with CP1A; lane 3, HAP2 core (ΔN154ΔC218) (14) plus CP1A; lane 4, cotranslated full-length HAP2 and HAP2 core with CP1A. Arrow indicates the gel loading origin.

consistent with our mutational study, we predict that the residues in the *a*, *d*, *e*, and *g* positions of an α-helix in the HAP2 SAD would mediate protein–protein interaction (Fig. 5). However, there is an apparent difference between HAP2 and the zipper proteins. The zipper proteins typically feature five hydrophobic residues at both the *a* and *d* positions. A zipper–zipper structure would therefore use 10 amino acid–amino acid interactions at these positions (4). The SAD of HAP2 could not derive an equivalent amount of interaction energy from the *a* and *d* positions because the helix is much shorter and there are only two mutationally sensitive residues at these positions (black in Fig. 5). We postulate that interactions at the *e* and *g* positions must be equally important for the stability of interaction in the HAP complex. These positions also contain two mutationally sensitive residues each. A coiled-coil structure for this domain and an interacting partner would allow residues at all four positions to interact with an α-helical partner.

Are there other important determinants of the SAD structure that were not revealed by the mutational study? There is a striking alignment of tyrosines at the *f* position and hydrophobic residues at the *b* position, as well as the positively charged lysines at the *c* position in the SAD (Fig. 5). Studies with synthetic peptides containing leucines and lysines showed that stability of an α-helix was enhanced by a spacing that placed leucine residues consecutively at the same position in the helix (25). In contrast, random placement of the leucine and lysine residues did not support formation of an α-helix (Table 2). By analogy, we imagine that the residues at the *b*, *c*, and *f* positions in the SAD help stabilize the α-helix. While mutations in these residues were not found, it is possible that multiple changes at these positions would be required to elicit a mutant phenotype by destabilizing the α-helix. A possible role for the lysines that align at the *c* position is less clear, but our experiments show that K166 and K173 are not mutationally sensitive (see Table 2).

We hypothesize that the mutationally sensitive residues of the HAP2 SAD directly contact the partner surface in the HAP complex. These residues form a small patch with the three charged arginine residues toward the C end. It is perhaps surprising that such a small region of a protein could mediate specific and stable protein–protein interaction with a partner. It is possible that the residues spanning Y176–E182, rigidly conserved in HAP2 homologs from divergent organisms, also contribute, albeit more weakly, to the interaction between HAP2 and its partner.

What is the partner that interacts with the SAD of HAP2? Our studies suggest that there is but one molecule of HAP2 per HAP complex, indicating that the partner is a different subunit in the complex. A prime candidate is, of course, HAP3. Genetic analysis of HAP3 has identified a region of that protein that is involved in DNA binding and a second region required for assembly or stability of the HAP complex (10). However, we have not demonstrated that HAP2 and HAP3 interact directly, using bacterially produced proteins (data not shown). Given this finding and the fact that the HAP complex has not been purified to homogeneity, we must consider the possibility that there is another, yet unknown, protein subunit in the HAP complex that binds to the HAP2 SAD, as proposed for the mammalian complex (26). This unknown protein may contain an α-helical motif with negatively charged residues which interact with the positively charged residues in the SAD.

The remarkable stability and specificity of the HAP2 SAD illustrate that a profound amount of structural information can be built into an extremely short amino acid sequence. It remains to be seen whether motifs as short as 12 aa will be generally important in determining protein–protein interactions in biological systems.

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