Valine 181 Is Critical for the Nucleotide Exchange Activity of Human Mitochondrial ADP/ATP Carriers in Yeast†

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ABSTRACT: We isolated yeast *Saccharomyces cerevisiae* cells transformed with one of the three human adenine nucleotide carrier genes (*HANC*) that exhibited higher growth capacity than previously observed. The *HANC* genes were isolated from these clones, and we identified two independent mutations of *HANC* that led to replacement of valine 181 located in the fourth transmembrane segment by methionine or phenylalanine. Tolerance of this position toward substitution with various amino acids was systematically investigated, and since *HANC/V181M* was among the most efficient in growth complementation, it was more extensively studied. Here we show that increased growth capacities were associated with higher ADP/ATP exchange activities and not with higher human carrier amount in yeast mitochondria. These results are discussed in the light of the bovine Ancp structure, that shares more than 90% amino acid identity with Hancps, and its interaction with the lipid environment.

Mitochondrial adenine nucleotide carriers (Ancps)[1] are nuclear encoded proteins of 30−35 kDa located in the mitochondrial inner membrane (MIM). They exchange cytoplasmic ADP^{3-} for mitochondrial ATP^{4-} under conditions of oxidative phosphorylation and therefore are key components of cell bioenergetics. We specially focus on the study of the three human *HANC* due to their involvement in various pathologies. Indeed, unusual *HANC* transcription patterns are associated with cancers or myopathies (*1*−*3*), and direct alterations of HAncp activity are induced by antibody binding in autoimmune pathologies. Recently, mutations of *HANC1* have been identified in several families of patients and in one sporadic patient presenting autosomal dominant progressive external ophthalmoplegia (adPEO) (*4*, *5*). This human disease shows a mendelian inheritance pattern. Among the four mutations reported (Ala114Pro, Val289Met, Leu98Pro, and Asp104Gly) only Ala114Pro was indirectly shown to impair HAncp1 activity, whereas the effects of the three other mutations were not evaluated (*4*). Furthermore, in the absence of structural data of these mutant proteins no assumption can be done on the consequences of these mutations on HAncp dimerization, which is compulsory to Ancp function. Structural studies need a highly efficient production of human carriers. We bypassed human biopsies, which usually give too low amounts of material, by setting up a system of heterologous expression of *HANC* in the yeast strain *JL1-3Δ2*, of which the endogenous *ANC* genes were deleted or disrupted as described in ref *6*.

The three *HANC* were able to complement *JL1-3Δ2* growth in the presence of a nonfermentable carbon sources such as glycerol or lactate. The ADP/ATP exchange properties of the three human carriers could be determined and have been shown to be different for each isoform. The growth rate was slow and the amount of HAncp in the mitochondrial membrane not sufficient to foresee structural studies. In addition, it was not possible to directly assess the function of the Ala114Pro or Val289Met HAnc1p variants associated with adPEO (*4*) because these proteins could not be detected in yeast (*6*).

In principle, a more efficient production of HAncp in *JL1-3Δ2* can be obtained by two means: mutations of the host cells or mutations of the *HANC* genes. Both could supposedly confer a more efficient import of the human carriers in yeast mitochondria, for example.

Therefore, we selected yeast cells transformed with each *HANC* that developed more rapidly on rich lactate medium (less than 13 days) due to UV or spontaneous mutagenesis. We identified two mutations, both modifying the same amino acid, valine 181, which was changed either into phenylalanine in HAnc1p or into methionine in HAnc2p. Val181 is

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[1] Abbreviations: adPEO, autosomal dominant progressive external ophthalmoplegia; Ancp, mitochondrial adenine nucleotide carrier protein; *ANC*, mitochondrial adenine nucleotide carrier encoding gene; ATR, atractyloside; B, beef; D_{600}, attenuance at 600 nm; H, human; MCF, mitochondrial carrier family; MIM, mitochondrial inner membrane; *Sc*, *Saccharomyces cerevisiae*; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMS, transmembrane segment; YPD, rich yeast extract peptone dextrose medium.

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located in the fourth transmembrane segment (TMS). Tolerance of this position toward various amino acids was investigated by site-directed mutagenesis, leading us to focus on the V181M variant to demonstrate that this position is critical for HAncp function in yeast.

MATERIALS AND METHODS

Strains, Media, and Transformation. Escherichia coli strain used for plasmid propagation was XL1-Blue [*recA1 endA1 gyrA96* (*Nal*^{r}) *thi hsdR17* (r_K^- m_K^+) *supE44 relA1 lac*$^-$ [*F′ Tn10* (*tet*^{r}) *proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]]. Bacterial strains were transformed according to standard methods either with calcium chloride (*7*) or by electroporation. *Saccharomyces cerevisiae* strain *JL1-3Δ2* (*MATα leu2-3,112 his3-11,15 ade2-1 trp1-1 ura3-1 can1-100 anc1::LEU2 Δanc2::HIS3 anc3::URA3*) was cultivated as described in ref *6*. Glucose-rich medium (YPD) was supplemented with 0.2% Tween 80 and 12 μg/mL ergosterol for cultivation under anaerobiosis. Yeast transformation was carried out by the lithium chloride method (*8*).

Chemicals. [^{3}H]Atractyloside (ATR) was synthesized as previously described (*9*). Protein concentration was determined using the bicinchoninic acid reagent kit from Sigma.

Cloning of the D3A/V181M Double Mutant. All the *ANC* genes used in these studies were cloned under the regulatory sequences of *ScANC2* (*5′Sc* and *3′Sc*) into the multicopy plasmid pRS424 as detailed in ref *6*. The plasmid pCM401/D3A, containing the *HANC1/D3A* ORF, was digested with *Kpn*I and *Bgl*II to isolate a 1.8 kbp fragment containing the *5′Sc* promoter and the 5′ end of *HANC1/D3A* ORF (*6*). This fragment was introduced in the pCM401/V181M vector digested with the same enzymes. The *Bgl*II site is located just upstream of the V181 codon, and therefore *HANC1* contains both mutations in the resulting plasmid, pCM401/D3A/V181M.

UV and Site-Directed Mutagenesis. JL1-3Δ2 transformed with *HANC1* was mutagenized by UV irradiation as described (*10*). Treated cells were plated (10^6 cells/plate), and mutants were selected onto rich lactate medium (YPLact) plates after 5 days of growth at 28 °C. Site-directed mutagenesis of *HANC* was performed using the Chameleon double-stranded kit (Stratagene) with the following mutagenic primers (mutated bases are underlined): ^{426}5′-GGTTTCAACGTCTCTATGCAAGGCATCATTATC-3′ ^{558} to mutate valine 181 into methionine in *HANC1*; ^{426}5′-GGCTTTAACGTGTCTATGCAGGGTATTATCATC-3′ ^{558} to mutate valine 181 into methionine in *HANC3*. The mutations leading to the substitution of valine 181 to glycine, alanine, aspartate, glutamate, arginine, tyrosine, and tryptophan were introduced in *HANC1* with the following degenerated oligonucleotide: 5′-GGTTTCAACGTCTC-TNNSCAAGGCATCATTATC-3′. A specific oligonucleotide was used to introduce all other substitutions. The codons used were TTG for leucine, ATT for isoleucine, AAA for lysine, CCA for proline, CAA for glutamine, ACT for threonine, TGT for cysteine, TCT for serine, AAT for asparagine, and CAT for histidine. The mutated *HANC* genes were subcloned into a multicopy plasmid under the control of *ScANC2* regulatory sequences as described in ref *6*.

Northern Blot Analyses. Total yeast cellular RNA was prepared and analyzed as described in ref *11*. *HANC1* RNA was hybridized with an antisense ^{32}P-labeled oligonucleotide probe located in the *HANC1* coding sequence (^{291}5′-CT-GCTTGTACTTGTCCTTGAAGGCGAAGTT-3′^{262}). The actin gene was hybridized with a [*Xho*I−*Hin*dIII] fragment corresponding to nucleotides 1762−2774. Hybridized probes were visualized with a PhosphorImager.

Other Methods. The protocols and materials used to perform isolation of mitochondria, cytochrome content determinations, ADP/ATP transport, [^{3}H]ATR binding measurements, and protein immunodecoration are described in ref *6*. Antibodies directed against Ade13p were a generous gift of Bertrand Daignan-Fornier (IBGC, Bordeaux, France).

RESULTS

Complementation of JL1-3Δ2 Cells Is Improved by V181 Mutation of HANC. In a previous work, we showed that *JL1-3Δ2* cells that are deficient for *ANC* activity could grow in the presence of lactate or glycerol when expressing any of the three human *ANC* (*6*). Moreover, the cells were "adaptable" when they were successively reinoculated in a fresh lactate-containing medium: the lag phase disappeared and the doubling times were reduced by a factor of around 2 for all of the *HANC* isoforms expressed. *HANC3* was the most efficient isoform to restore yeast growth, and HAnc3p presented the highest V_{max} and K_M^{ADP}. HAnc1p and HAnc2p presented similar kinetic constants, whose values were consistent with *HANC1* and *HANC2* lower capacities to restore yeast growth. However, the *HANC* genes remained less efficient than *ScANC2*. That could be partly due to the low HAncp content in yeast mitochondria (*6*). Therefore, we searched for *JL1-3Δ2* cells transformed with *HANC* that developed more efficiently on YPLact either spontaneously (*HANC1*, *HANC2*, or *HANC3*) or after UV mutagenesis (*HANC1*). Such clones were likely to have an increased HAncp content.

After UV mutagenesis, 43 fast growing clones appeared on YPLact plates after 5−6 days at 28 °C instead of the 11−13 days needed for wild-type *HANC*. Besides, a clone with a spontaneous improved growth was also isolated from *JL1-3Δ2* cells expressing *HANC2*. While usually all clones are white on minimal glucose medium minus tryptophan, this clone was red-colored. This color reflects a higher respiratory metabolism (*12*) and thus possibly a higher ADP/ATP exchange activity of HAnc2p. Plasmid DNA was isolated from this clone and from another one that developed significantly faster than the others after UV mutagenesis. Both *HANC* genes were subcloned into fresh plasmids and thereafter were used to back-transform *JL1-3Δ2* to determine if the observed phenotype was due to *HANC* or *JL1-3Δ2* mutations. Because cell growth was faster after back-transformation than with the wild-type *HANC*s, we could conclude that the observed phenotypes were due to one or several mutations in the *HANC* ORFs. They were sequenced, and strikingly, we identified mutations of the same codon corresponding to valine 181. This residue was changed into phenylalanine in HAnc1p (G541 → T in the *HANC1* gene) and into methionine in HAnc2p (G541 → A in the *HANC2* gene). The other mutants obtained after UV treatment are currently under analysis. This paper focuses on the characterization of valine 181 mutants.

This residue is located in the fourth TMS and is conserved throughout the known Ancp sequences, except for plants

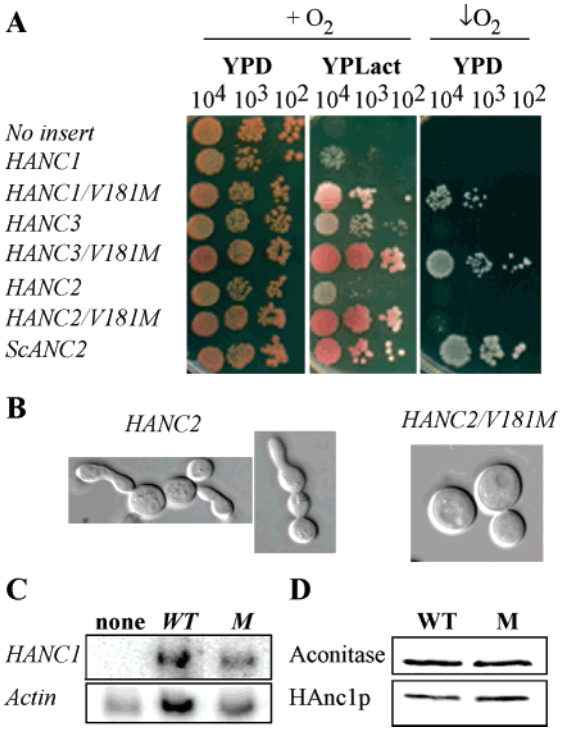

FIGURE 1: (A) Growth phenotypes of *JL1-3Δ2* conferred by the *V181M* mutation. Cultures of *JL1-3Δ2* cells expressing the wild-type *HANC* or the mutants *HANC/V181M* were diluted and plated (10^4 to 10^2 cells) onto rich medium under normal ($+O_2$) or reduced oxygen concentration ($\downarrow O_2$). The medium contained lactate (YPLact), glucose (YPD), or glucose supplemented with 0.2% Tween 80 and 12 μg/mL ergosterol ($\downarrow O_2$, YPD). Growth was followed at 28 °C for 3 days (YPD) or 13 days (YPLact and $\downarrow O_2$, YPD). (B) The *V181M* mutation restores normal cell morphology. Cells expressing *HANC2* and *HANC2/V181M* were cultivated in YPLact, collected during log phase, washed with distilled water, and observed under a light microscope. (C) *HANC1* and *HANC1/V181M* are transcribed similarly. Total RNA was isolated from *JL1-3Δ2* cells expressing *HANC1* (WT) or *HANC1/V181M* (M) and cultivated in YPLact. 30 μg was loaded in each lane. After transfer onto a nylon membrane, mRNA was hybridized with an antisense oligonucleotide probe for *HANC1* or the actin gene (upper panel and lower panel, respectively). (D) Immunodetection of wild-type and mutant HAnc1p in isolated yeast mitochondria. Mitochondrial proteins (10 μg in each lane) were subjected to SDS−PAGE (12.5% acrylamide). After transfer onto a nitrocellulose membrane, proteins were immunodecorated with a polyclonal antibody raised against aconitase (upper panel) or directed against a synthetic peptide of HAnc1p (lower panel).

where it is a cysteine residue (*13*). The V181M mutation was introduced by site-directed mutagenesis in the two other *HANC*s, *HANC1* and *HANC3*, to evaluate if the growth phenotype was isoform dependent. The variant ORFs were then cloned in a multicopy vector (see Materials and Methods), and the resulting plasmids were used to transform *JL1-3Δ2*.

The cells expressing either one of the *HANC/V181M* genes developed rapidly on YPLact (5 days; Figure 1A). Unlike what was observed for the wild-type *HANC*s, the three *HANC/V181M* variants restored growth at the same level. In liquid YPLact medium, the doubling time was about 10 h whatever the *HANC/V181M* isoform expressed. It was also constant throughout successive inoculations in YPLact, indicating that the transformants did not exhibit the "adaptation" phenomenon described for the wild-type *HANC* (*6*). The cell yield was doubled in the presence of *HANC/V181M* ($D_{600} = 8$ compared to 4 for *HANC*), and no lag phase was observed before growth started.

Wild-type *HANC*s could not sustain *JL1-3Δ2* growth under anaerobiosis on rich glucose medium plates, while the three *HANC/V181M* did it but behaved differently (Figure 1A): *HANC3/V181M* was the most efficient and *HANC2/V181M* could barely develop under these conditions. Actually, a similar efficiency order was observed on YPLact plates for the cell expressing the various wild-type *HANC*s (*6*), pointing out again differences in HAncp properties depending on the isoform.

Besides its crucial effect on yeast growth, the *V181M* mutation modified noticeably the cell morphology. Indeed, cells expressing *HANC*s appeared of smaller size under the microscope when compared to wild-type cells, tended to aggregate, and divided with difficulties (Figure 1B). In contrast, cells expressing *HANC2/V181M*, for example, were larger and divided normally. These morphological features resembled those of cells expressing *ScANC2* (Figure 1B).

Cell dry weights per attenuance unit at 600 nm were similar for the wild-type *HANC2* and the *HANC2/V181M* cells (0.81 and 0.76 mg, respectively) as well as the mitochondria contents. Those were determined by immunodecoration of equivalent amounts of cell extracts with antibodies directed against porin, a mitochondrial protein, and Ade13p, a cytosolic protein. The signal intensities were quantified, and the porin/Ade13p ratios were 16.3 arbitrary units (AU) for the *HANC2* cells and 18.5 AU for the *HANC2/V181M* cells. These results indicated that the V181M mutation did not increase significantly mitochondria biogenesis that could have explained a more efficient growth of the mutant cells on YPLact.

Tolerance of Position 181 toward Amino Acid Substitutions. Since two very different amino acid residues at position 181, methionine and phenylalanine, could significantly improve in vivo HAncp function, it was important to determine if any amino acid could replace valine at position 181 with a similar effect. Therefore, we introduced the other 17 residues by site-directed mutagenesis of *HANC1*. *JL1-3Δ2* transformants were tested for their ability to grow on various media when expressing the *HANC1/V181X* variants.

As shown in Figure 2 and based on growth on YPLact, we could divide the transformants, and therefore the amino acids, into three groups (I to III). In group I, they developed similarly to *HANC1* transformants (Cys, Ser, His, Asn, and Ile) or slightly better (Ala and Gly). In group II, they developed much better (Leu, Thr, Gln, Met, and Phe), and in group III, transformants developed barely (Trp) or not at all (Glu, Asp, Lys, Arg, Pro, and Tyr). All transformants could develop under anaerobiosis in group II, whereas only two in group I, Ala and Gly, could develop. Phe and Met were the most efficient to restore growth on YPLact. Cells expressing *HANC1/V181F* had a doubling time of about 9 h in liquid YPLact, and there was no lag phase (Table 1). The growth yield was not modified for the cells expressing *HANC1/V181F* as compared to *HANC1* but was slightly lower than when expressing *HANC1/V181M* (Table 1). Thus, these two amino acids may not be fully equivalent at position 181. These results show that position 181 in HAnc1p can tolerate various amino acids, but charged residues, two aromatic residues, tyrosine and tryptophan, and proline, probably due to the structural constraints it imposes. Surpris-

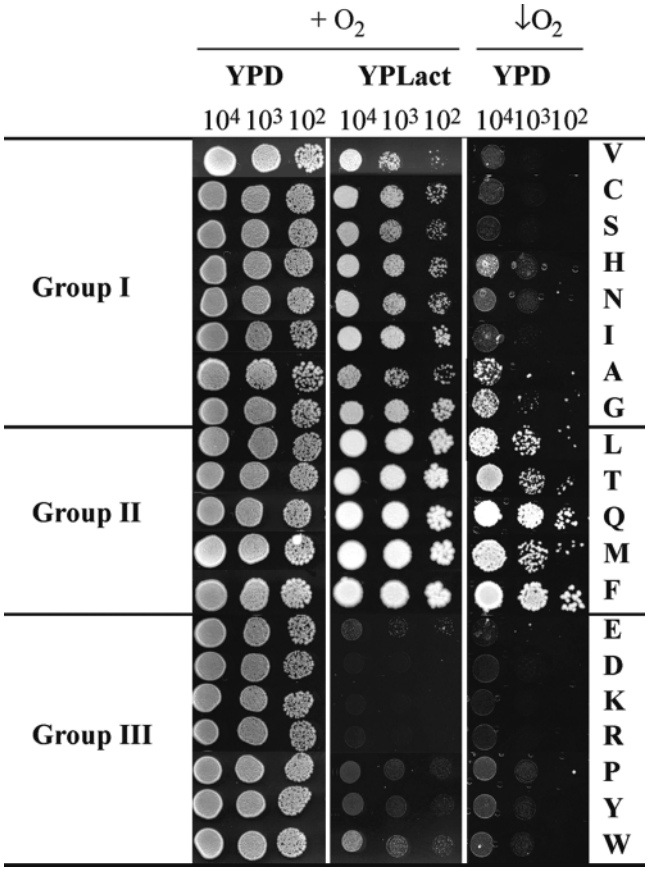

FIGURE 2: Effect of V181 substitutions on growth of cells expressing *HANC1*. Cultures of *JL1-3Δ2* cells expressing wild-type or variant *HANC1* were diluted and plated (10^4 to 10^2 cells) on a rich medium containing glucose or lactate (YPD or YPLact). Cells were cultivated at 28 °C under normal (+O_2, YPD, YPLact) or reduced oxygen concentration (↓O_2) for 3 days (YPD) or 13 days (YPLact and ↓O_2, YPD). The amino acid code used is the one-letter one. Complementation groups I, II, and III are described in the text.

Table 1: Comparison of Growth Properties of *JL1-3Δ2* Expressing Various *HANC1*

	lag phase[a]	doubling time[a]		growth yield[b]
HANC1 gene	nonadapted[c]	nonadapted[c]	adapted[c]	adapted or not[c]
WT[d]	55	36	17	4
V181M	none	10	10	8
V181F	none	9	9	7
D3A/V181M	70	35	17	7

[a] In hours. [b] D_{600} was measured during the stationary phase of cultures. [c] Transformants were cultivated in YPLact at 28 °C after overnight preculture in synthetic complete glucose medium minus tryptophan (nonadapted) or after three successive precultures in YPLact (adapted). [d] Determined in ref *6*.

ingly, five residues larger than valine can substantially improve in vivo HAnc1p function.

V181M Mutation of the Wild-Type Carrier Increases Its ADP/ATP Exchange Activity but Not Its Amount in Mitochondria. Since the V181M and V181F substitutions were the most efficient, we chose to get further insights into the properties of the V181M variant. To investigate whether this mutation has a role on *HANC* expression or HAncp function, we first examined the *HANC1/V181M* transcription level. It was similar for *HANC1/V181M* and *HANC1* (Figure 1C) and therefore could not account for an improved yeast growth rescue by *HANC1/V181M*.

The level of HAnc1p/V181M in mitochondria was then examined by immunodecoration of mitochondria lysates with an antibody raised against a HAnc1p synthetic peptide. No significant difference was observed between the amounts of HAnc1p and HAnc1p/V181M (Figure 1D). To quantify more accurately HAncp contents, we performed [^{3}H]ATR binding experiments with isolated mitochondria. Due to its high specificity and affinity for Ancp, the maximum number of ATR binding sites will reflect the amount of functional transporter present in the mitochondrial membrane. There was a small decrease (30%) of the amount of HAnc1p/V181Mp compared to wild-type HAnc1p in yeast mitochondria, while the cytochrome *b* content did not change significantly (Table 2). As a result, the carrier amount in the MIM cannot account for the phenotype observed with the *V181M* variant.

The adenine nucleotide contents were measured by bioluminescence for both mitochondria types (data not shown). The ADP amounts were similar, and the ATP content was slightly increased ($\leq$25%) for the V181M variant compared to the wild-type HAnc1p. As a consequence, the ATP/ADP ratio was slightly increased for the V181M variant (2.3 versus 1.9). However, the overall amount of AMP + ADP + ATP was the same for both mitochondria types. We therefore determined the ADP/ATP exchange activity of the V181M variant and compared it to HAnc1p. HAnc1p/V181M-containing mitochondria were incubated with fixed concentrations of ADP, and the nucleotide exchange activity of HAncp was measured as described in ref *6*. As seen in Table 2, the K_M^{ADP} values are similar for the wild-type HAnc1p and the V181M variant. In contrast, the V_{max} value for HAnc1p/V181M is about 3.5 higher than for HAnc1p [110 versus 34 nmol of ADP min^{-1} (mg of protein)$^{-1}$]. Thus, a methionine instead of a valine at position 181 can substantially improve the ADP/ATP exchange activity of HAnc1p.

V181M Can Rescue Mutations That Impair HANC1 Function in Yeast. Since the V181M substitution has such important effects at the cellular and molecular levels, we hypothesized it could overcome the negative effect of an amino acid substitution that results in a defective HAnc1p function in vivo. We have introduced *V181M* in *HANC1/D3A* and *HANC1/A114P*, which were previously shown to preclude growth of *JL1-3Δ2* on YPLact but not on glucose medium (*6*). Moreover, these two HAnc1p variants could not be detected in yeast cell extracts. We now observe that *JL1-3Δ2* cells expressing *HANC1/D3A/V181M* and *HANC1/A114P/V181M* were able to develop on YPLact (Figure 3A). The doubling time of the former strain was equivalent to that of the cells expressing *HANC1*, and the growth yield was higher (D_{600} = 8 compared to 4 for *HANC1*; Table 1). The amounts of HAncp in isolated mitochondria were determined by immunodecoration and [^{3}H]ATR binding experiments. Whereas HAnc1p/A114P/V181M and HAnc1p were present in similar amounts (0.22 as compared to 0.25 mol of ATR/mol of cyt *b*), the amount of HAnc1p/D3A/V181M was one-third of HAnc1p (Table 2 and Figure 3B). When the ADP/ATP exchange activities were examined, the V_{max} values of the double mutants were comparable or twice that of HAnc1p (Table 2). In contrast, the K_M^{ADP} value of HAnc1p/A114P/V181M was doubled, whereas that of HAnc1p/D3A/V181M was close to that of HAnc1p.

Table 2: Kinetic Parameters of [^{3}H]ATR Binding and ADP/ATP Exchange by HAncp in Isolated Yeast Mitochondria[a]

	ATR binding				ADP/ATP exchange	
HAnc1p variant	K_d^{ATR} (nM) [b]	ATR_{max} (pmol/mg of protein)[b]	cyt *b* (pmol/mg of protein)	ATR_{max} (mol of ATR/ mol of cyt *b*)	K_M^{ADP} (μM)	V_{max}^{ADP} [nmol min^{-1} (mg of protein)$^{-1}$][c]
WT	34	174	684	0.25	3.7	33
V181M	32	123	650	0.18	3.3	110
D3A/V181M	49	74	883	0.08	4.5	57
A114P/V181M	109	117	522	0.22	8.4	30

[a] [^{3}H]ATR binding and ADP/ATP exchange were measured with mitochondria isolated from *JL1-3Δ2* expressing *HANC* genes (wild type or mutants). The values given are the averages of at least two experiments. [b] The standard error value was no more than 20−25%. [c] The standard error value was no more than 10−15%.

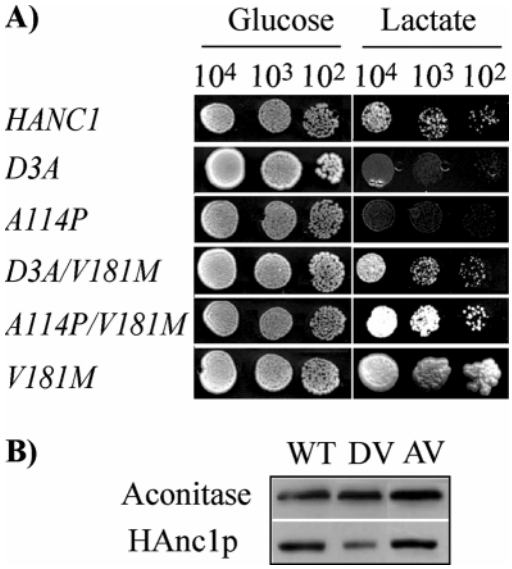

FIGURE 3: (A) *V181M* mutation rescues the growth defect induced by *D3A* or *A114P* mutations. Cells expressing the various *HANC* variants were cultivated overnight in YPLact. They were then diluted (from 10^4 to 10^2 cells) and plated on rich medium containing glucose or lactate. Growth was followed at 28 °C for 3 days (Glucose) or 13 days (Lactate). (B) Immunodetection of HAnc1p variants in yeast mitochondria. DV and AV stand for HAnc1p/D3A/V181M and HAnc1p/A114P/V181M, respectively. 10 μg of mitochondrial proteins from both strains was loaded on an SDS−polyacrylamide gel. After transfer onto a nitrocellulose membrane, immunostaining with the anti-HAnc1p antibodies was carried out as described in Materials and Methods. The aconitase was stained with a specific antibody as an internal control of the protein amount loaded.

DISCUSSION

Structural characterization of HAncp, wild type or mutant, implies that it can be produced in sufficient amounts. This was not satisfactorily achieved through the heterologous expression system we set up in a previous work (*6*). For this purpose, our approach was to isolate *JL1-3Δ2* clones expressing each of the three *HANC* that were able to grow faster on a nonfermentable carbon source. Two types of mutants were obtained: *JL1-3Δ2* mutants and *HANC* mutants. Here we describe the characterization of two *HANC* ORF mutants. Both were mutated in codon 181, emphasizing the potential role of this residue in HAncp production or activity. The other mutants are currently under characterization.

Valine 181 is located in the middle of the fourth TMS of HAncp. Its replacement with a methionine in each HAncp isoform improved substantially cell morphology and growth characteristics in YPLact as well as under anaerobiosis. Although the three *HANC/V181M* isoforms restored a similar growth on YPLact, the complementation level under anaerobiosis was dependent on the isoform *HANC/V181M*. The efficiency order for the three isoforms was then similar to that observed on YPLact for wild-type *HANC* expressing cells. In the absence of oxygen, mitochondria have to import cytosolic ATP in exchange for matrix ADP. This electrogenic exchange, coupled to ATP hydrolysis, contributes to generation of a membrane potential, which, although lower than under aerobic conditions, is sufficient for mitochondria maintenance (*14*). Furthermore, the electrogenic nature of the ADP/ATP exchange makes it membrane potential dependent. This might explain that differences between the three human isoforms become apparent again when the membrane potential is lowered, such as under anaerobiosis.

A valine at a position equivalent to 181 is conserved in yeast and animal Ancp, with very few exceptions (isoleucine in *Halocynthia roretzi* and cysteine in *Trypanosoma brucei*). In plant, this valine is replaced with a cysteine. To get further insights in the role of this residue in HAnc1p, we individually introduced at position 181 all amino acids other than valine. Only seven residues could not substitute for valine and were not able to rescue yeast growth. They correspond to the four charged amino acids (Lys, Arg, Asp, Glu), two aromatic residues (Tyr, Trp), and proline (Pro) that affects helix continuity. None of these residues is naturally occurring at position 181 of Ancp (see above). All other amino acids are able to rescue yeast growth at the same level as valine (Cys, Ser, His, Asn, Ile, Ala, Gly) or even better (Leu, Thr, Gln, Phe, Met), with Cys and Ile naturally occurring in Ancp homologues. Since two residues with a similar chemical group in the lateral chain restored different growth properties (serine versus threonine or asparagine versus glutamine, for example), we could suggest that the chemical nature of the residue at position 181 is not important. More likely, the residue size could influence growth level, as threonine and glutamine are more efficient than serine and asparagine, respectively. However, alanine and glycine are smaller residues than valine but at least as effective. Methionine is more efficient than cysteine. Therefore, the mobility of the lateral chain could also be important. Aromatic residues afford an exception since the hydroxyl group of tyrosine is deleterious to HAncp function as compared to phenylalanine that is more effective than valine. However, as V181 is largely conserved in Ancp sequences, the effect of its replacement with other amino acids is likely to depend also on residues in close vicinity. Those are subject to variation (position 181 ± 2), but their interactions with V181 are

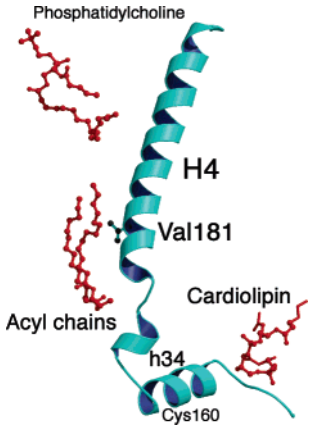

FIGURE 4: 3-D structure of BAncp1 [adapted from 1OKC.pdb (*15*)] restricted to amino acids 150−200. They encompass the fourth transmembrane segment (amino acids 177−200) and part of the second matrix loop (amino acids 150−176). The amino acid numbering starts from Met1 of the BAnc1p sequence deduced from *BANC1*. Valine 181 points to acyl chains (in brown on the left side) that putatively belong to a cardiolipin molecule. Cys160, located in the small helix h34 of the second matrix loop, is close to another cardiolipin molecule, represented in brown on the right side of the drawing. Cys160 occupies a position of BAncp1 similar to that of Cys57 in the first matrix loop. This last residue was described as important for cardiolipin binding (*16*). The phosphatidylcholine molecule facing toward Ala114 is represented in brown in the upper left side of the drawing.

complex and difficult to analyze. They will be the focus of other studies.

When the ADP/ATP exchange activity of HAnc1p/V181M was further investigated, we found that the V_{max} value was strongly increased, whereas the K_M^{ADP} value was unchanged between the wild type and the mutant. The slight increase of the mitochondrial ATP/ADP ratio for the V181M variant does not account for the 3-fold increase of the V_{max} value. Consequently, V181 may not be involved in direct interaction with nucleotides but may be important for HAncp structure or conformational changes during nucleotide transport.

Recently, crystal structure of the monomer of bovine Anc1p (BAnc1p) was obtained at 2.2 Å resolution (*15*). Since BAnc1p and HAnc1p share 96% identity, it is reasonable to assume that both proteins will adopt the same three-dimensional structure. On the basis of the crystal structure of BAncp, V181 is located near the ligand binding cavity, but oriented in the opposite direction toward two acyl chains that could belong to a cardiolipin molecule of the lipid bilayer (ref *15* and Figure 4). Cardiolipins have been shown to be important for the yeast ADP/ATP carrier activity, and one cysteine residue is supposed to play a crucial role in these interactions for the yeast (Cys73) and bovine (Cys57) carriers (*16*, *17*). Our result suggests that the residue at position 181 could stabilize the carrier in the mitochondrial membrane via its interactions with surrounding lipids, particularly with cardiolipins. Beef and yeast mitochondria present different lipid compositions (*18*) that may account for the fact that the human Ancp are not perfectly adapted to the yeast lipid environment and have to mutate to do so. In yeast mitochondria, sterol (ergosterol versus cholesterol in animals) is localized to the inner membrane in contrast to mammals where cholesterol is localized mainly to the mitochondria outer membrane and almost absent in the inner membrane (*19*, *20*). In addition, two main features distinguish the yeast mitochondria lipids from that of bovine heart mitochondria: (i) cardiolipin represents only 13% of yeast mitochondria phospholipids versus 20%; (ii) the unsaturation index of cardiolipin is much lower in yeast than in beef heart (*18*). When we introduced the *V181M* mutation in the equivalent position in the yeast *Sc*Anc2p, *V197M*, no difference was observed for yeast growth on YPLact (data not shown). Likewise, when an amino acid residue leading to inactive HAnc1p, namely, tyrosine, was introduced at position 197 of *Sc*Anc2p, the cells expressing *ScANC2/V197Y* were able to grow as well as with the wild-type *ScANC2*. These observations indicate that position 181 of HAncp is not functionally equivalent to position 197 of *Sc*Anc2p. Thus, the phenotype obtained with the single *V181M* mutation could be specific of the yeast heterologous expression system. Although mouse *ANC1* knock-out cells (*21*) are available to test this hypothesis, this system may not be suitable since overexpressing mouse *ANC1* into human cells induced apoptosis (*22*). Another possibility is to manipulate the yeast lipid composition either by changing the carbon source of the growth medium or the growth temperature or by using specific mutants of the cardiolipin and ergosterol biosynthetic pathways. These possibilities will be used in future work.

The *V181M* mutation in HAnc1p has a strong beneficial effect on the carrier function and as a consequence on yeast growth rescue. We thus used this mutation to indirectly evaluate the effect of inactivating mutations such as *D3A* and *A114P*. *D3A* was supposed to impair HAncp import (*6*), and *A114P*, found in patients presenting adPEO, was proposed to disturb the carrier's structure (*4*). None of the variant carriers could restore yeast growth and could be detected in yeast. The double mutants *HANC1/D3A/V181M* and *HANC1/A114P/V181M* were generated and expressed in yeast. *V181M* in combination with either mutation was able to rescue growth of the transformants on YPLact. The HAnc1/D3A/V181Mp protein levels were strongly reduced as compared to the wild-type HAnc1p and the V181M variant. This is in agreement with our suggestion that *D3A* mutation could impede HAncp import into MIM (*6*). Therefore, for the double mutant, higher ADP/ATP exchange activity compensated for lower HAncp content, but affinity for ATR binding was similar to the HAnc1p one. The HAnc1/A114P/V181Mp nucleotide exchange rate was similar to that of HAnc1p, but in contrast, the K_d^{ATR} and the K_M^{ADP} values were affected by the presence of a proline residue at position 114. These results suggest that Ala114 might be involved in interactions with the nucleotides. However, since Ala114 is located in a region of Ancp probably exposed to the intermembrane space and directed opposite the nucleotide binding cavity (*15*), the effect of Ala114Pro is likely to be mediated through impaired conformational changes by disturbing the Ancp structure in the upper part of the helix extension of the third TMS. Indeed, in the bovine Ancp structure, Ala114 interacts with a phosphatidylcholine molecule (PC) (see The Protein Data Bank, accession number 1OKC), indicating that, in vivo, Ala114 is likely to interact with components of the lipid bilayer. Furthermore, this PC faces the two acyl chains that are close to Val181 (Figure 4). Altogether, these lipids would facilitate the dynamic interaction of Ancp with the lipid bulk phase. The lateral pressure it exerts would help in maintaining an efficient contact between the two protomers during the

catalytic cycle of the dimeric transporter. Conversely, mutation of Ala114 could prevent interaction with the tightly bound lipids, thereby loosening the interaction between protomers of Ancp. This would explain partial Ancp impairment by the Ala114Pro mutation that would be partially reversed by Val181 mutations, allowing tighter interactions with environmental lipids.

In this paper, we have successfully expressed the *HANC1/A114P* variant by introducing a mutation, *V181M*, that renders HAnc1p hyperactive in yeast. This stratagem will possibly allow to study in yeast the mutations that have been described associated with adPEO (*4*, *5*), which until now could not be expresssed in yeast.

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