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The Intriguing Evolution of the "b" and "G" Subunits in F-type and V-type ATPases: Isolation of the *vma-10* Gene from *Neurospora crassa*

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We have characterized the *vma-10* gene which encodes the G subunit of the vacuolar ATPase in *Neurospora crassa*. The gene is somewhat unusual in filamentous fungi because it contains five introns, comprising 71% of the region between the translation start and stop codons. The 5′ untranslated region of the gene contains several elements that have been identified in other genes that encode subunits of the vacuolar ATPase in *N. crassa*. A comparison of G subunits from *N. crassa*, *S. cerevisiae*, and animal cells showed that the N-terminal half of the polypeptide shows the highest degree of sequence conservation. Most striking is the observation that this region could form an alpha helix in which all of the conserved residues are clustered on one face. Subunit G appears to be homologous to the b subunit found in F-type ATPases. The major difference between the b and G subunits is the lack of a membrane-spanning region in the G subunit. We have also identified homologous subunits in the operons which encode V-type ATPases in a eubacterium, *Enterrococcus hirae*, and an archaebacterium, *Methanococcus jannaschii*. As in eukaryotic vacuolar ATPases the G subunit homologs lack a membrane-spanning region. Although the b and G subunits appear to be derived from a common ancestor, significant changes have evolved. In F-type and V-type ATPases these subunits can have zero, one, or two membrane-spanning regions and can also differ significantly in the number of copies per enzyme.

INTRODUCTION

The F-type and V-type ATPases are large multisubunit complexes, which couple the transport of protons to the synthesis or hydrolysis of ATP (Bowman and Bowman, 1996). These enzymes are ancient relatives that diverged from a common ancestor before the evolution of the eukaryotic cell (Gogarten *et al.*, 1989). The general shape and structure of F and V-type ATPases is similar. Several types of polypeptides are embedded in the membrane and form a proton conducting pathway. This portion of the enzyme is called the F_0 or V_0 sector. Protruding from the membrane is a ball and stalk structure, which contains the ATP binding sites (Senior, 1990; Dschida and Bowman, 1992). Composed of at least five different types of polypeptides, this sector is named F_1 or V_1. It can be dissociated from the membrane and still retain ATPase activity (Graf *et al.*, 1996; Crider *et al.*, 1997).

The stoichiometry of subunits and the size and shape in electron micrographs suggest that the overall structure of the F_1 and V_1 sectors is very similar (Bowman *et al.*, 1989; Dschida and Bowman, 1992). The two largest polypeptides in the F_1 ATPase (α and β) are clearly homologous to the largest polypeptides in the V_1 ATPase (A and B). However, identifying V_1 subunits that are homologous to the smaller polypeptides of F_1 has been difficult even though the sequences of most of these subunits have been determined.

The proton conducting portions of the F- and V-type ATPases may be more dissimilar. The F_0 sector of the enzyme from *Escherichia coli* has three type of subunits, a (30 kDa), b (17 kDa), and c (8 kDa) in a stoichiometry of 1:2:10–12 (Fillingame, 1990; Senior, 1990). Reconstitution experiments indicate that all

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three types are necessary to form a functional proton channel (Schneider and Altendorf, 1985). No homolog of the a subunit has been identified in a V-type ATPase. A much larger polypeptide (96 kDa) in the V_0 sector could have the analogous function in the V-type ATPase (Leng *et al.*, 1996). V-type ATPases also contain a polypeptide named subunit c, which is clearly homologous to the subunit c in F_0 (Mandel *et al.*, 1988). Although twice as large (16 kDa) it is present in only six copies per enzyme (Arai *et al.*, 1988). Thus the total number of membrane helices contributed by the c subunits is probably the same in both F- and V-type ATPases. Recent experiments with the V-ATPase from *Saccharomyces cerevisiae* indicate that the V_0 structure may be more complex. Three genes, *VMA3, VMA11*, and *VMA16*, encode homologs of subunit c and every V-ATPase appears to contain all three gene products (Hirata *et al.*, 1997).

The b subunit, the subject of this report, is a 146-residue polypeptide in *E. coli*. The 25 residues at the N-terminus form a hydrophobic membrane-spanning region, while the rest of the polypeptide has a high proportion of charged and polar amino acids. The polar segment appears to form an elongated helical structure, which binds to several subunits in the F_1 sector (Perlin *et al.*, 1983; Dunn, 1992; Collinson *et al.*, 1996). It has recently been suggested that the b subunit may be part of a stator to prevent the rotation of the $\alpha 3\beta 3$ complex during catalysis (Wilkens *et al.*, 1994; Cross and Duncan, 1996; Pedersen, 1996). It was therefore surprising to see recent reports of a V-type ATPase homolog of the b-subunit, named subunit G, that lacked a membrane-spanning domain (Supekova *et al.*, 1995, 1996) and that, in one report (Lepier *et al.*, 1996), appeared to be a constituent of the V_1 sector. We have isolated the gene encoding subunit G in *Neurospora crassa* and have found that comparison of the sequences from several different organisms suggests a structure for the N-terminal half of this polypeptide. The b subunit has evolved in a most interesting fashion, as shown by comparison of this polypeptide in F-type and V-type ATPases from eubacteria, achaebacteria, and eukaryotic cells.

MATERIALS AND METHODS

Purification of Vacuolar Membranes and the V_1 Sector of the Vacuolar ATPase

Vacuolar membranes were prepared as previously described (Dschida and Bowman, 1995). To isolate the V_1 sector of the vacuolar ATPase, membranes (1 mg/ml) were suspended in 100 mM NaNO3 for 15 min, then pelleted by centrifugation for 10 min at 15,000 × *g*. The washed membranes (100 μg) were resuspended in 100 μl of buffer (100 mM $NaNO_3$, 10 mM NH_4Cl, 5 mM NaATP, 5 mM $MgSO_4$, 10 mM PIPES, pH adjusted to 7.4 with Tris base), incubated on ice for 60 min, and pelleted by centrifugation for 10 min at 15,000 × *g*. The supernatant containing the V1 subunits was analyzed by polyacrylamide gel electrophoresis.

Purification of Polypeptides and Protein Sequencing

Polyacrylamide gels were stained with Coomassie Blue. The 18-kDa band was excised, electroeluted from the gel as previously described (Stone *et al.*, 1989), and digested with trypsin at a ratio of 1 part trypsin /10 parts protein. After an overnight incubation at 37°C a second aliquot of trypsin was added, and the protein was incubated for a further 2 hours before separation of the peptides by reverse-phase high-pressure liquid chromatography on a C18 (VYDAC 218TP) column. Peptides were sequenced using a Porton automated sequencer.

Isolation of the *vma-10* Gene

A 756-bp cDNA, described in Results and Discussion, was obtained from the Neurospora Genome Project at The University of New Mexico (Nelson *et al.*, 1997). This clone, named NC5G10-T7, was used to screen a *N. crassa* genomic library in the vector pMOcosX (Fungal Genetics Stock Center, University of Kansas, Kansas City, Kansas). Four cosmid clones hybridized strongly to the cDNA, G4-11E, G5-7C, G6-10C, and G6-12F. A 2.5-kb *Bam*H1 fragment of G5-7C was identified as containing the entire *vma-10* gene, subcloned into an M13 phage vector, and sequenced using the Applied Biosystems (ABI) PRISM dye terminator kit (Perkin Elmer, Foster City, California).

RESULTS AND DISCUSSION

Isolation of the *vma-10* Gene

The V_1 sector of the vacuolar ATPase was prepared as described in Materials and Methods and analyzed by electrophoresis on polyacrylamide gels. As

shown in Fig. 1 a prominent band was seen at an apparent molecular weight of 18 kDa. Tryptic peptides obtained from this band were sequenced by automated Edman degradation. None of the sequences obtained showed a high degree of similarity to any sequences in the Genbank database. However, a cDNA sequenced by the Neurospora Genome Project was identified that had regions of similarity to the *VMA10* gene product of *S. cerevisiae* (S. Kahn, D. Natvig, and M. A. Nelson, University of New Mexico, personal communication). The complete sequence of the cDNA was obtained and was found to encode four of the tryptic peptides from the 18-kDa band. The cDNA clone was used to isolate a 2.5-kb fragment of genomic DNA, which contained the entire gene, named *vma-10* (Fig. 2).

Chromosomal Mapping of the *vma-10* Gene

The chromosomal location of *vma-10* was determined, using genomic DNA digested with *Xho*1, by analysis of restriction fragment length polymorphisms (Metzenberg and Grotelueschen, 1992). The gene mapped to linkage group II between the *Ncr-2* locus and the H3H4 locus. Three other genes that encode subunits of the vacuolar ATPase, *vma-2*, *vma-6*, and *vph-1*, have been found on the same chromosome, separated from *vma-10* by 15–30 map units.

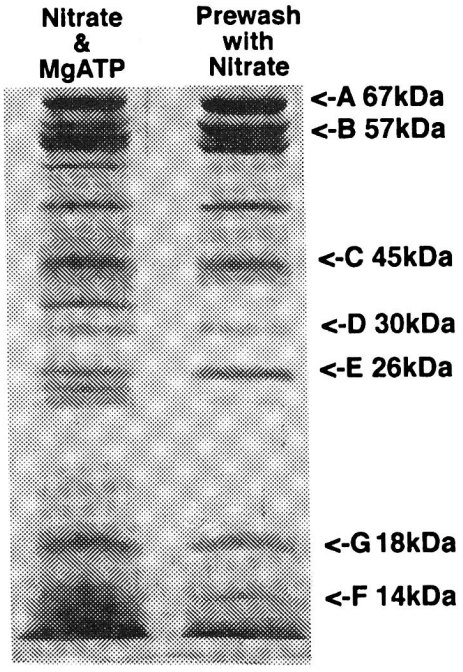

Fig. 1. Separation of polypeptides in the V1 sector of the vacuolar ATPase. As described in Materials and Methods, vacuolar membranes were either suspended directly in nitrate and MgATP (left lane) or washed once in nitrate without MgATP, then suspended in nitrate and MgATP. After centrifugation the supernatants were analyzed by polyacrylamide gel electrophoresis. The putative subunits of the vacuolar ATPase are identified with arrows.

Characteristics of the *vma-10* Gene

Five introns of 96, 283, 113, 238, and 159 bp were identified. Although introns are common in genes of filamentous fungi the number and size of the introns identified in *vma-10* was somewhat surprising. Between the translation start and stop sites 71% of the gene is composed of introns, the most for any gene isolated from *N. crassa*. The first intron is positioned immediately after the first codon, identical to the posi-

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1    GCAGCGGCAGGCCGGCTGCGGGCGGCCACTGTAACGCTGTGCAGGCGCGCCTCCTAGCGGGGGAGGTAGC
71   GGTACCGGGCTACGACGACGCCCGCCAACGGCCAACGCCAACAACCCACTTGACCTCAGGTCCCGTCGAC
                                                    ↓start of cDNA
141  CATCATCGCATCGCATCGCAGCGTATCGAATCATCGACGGGCCGCATCAACATCACGATATTACGACTGA
211  CACGAACAACAAGACCAACAATCCATACCTATACTCGACTCACTACGACCGATACCATCTCCACCTCCTA
281  ATCGCGATTGCTTCAGCCTCCGAACTCCGAAGCGATTAGCACCGCTACCTCTACCTAGCTCCGACTACCA
351  CCTCTATCTACCTCTACAGCTATCACCATGgttcgttcccagctagcatagcatcttcgtccgcaccgac
                                  M
421  gacgccaacataagaaagaataacgaaaagctaacgggatggatggttggaaatagTCGGCCCAAAAGTC
                                                          S  A  Q  K  S
491  AGCGGGTATCCAGCTTCTTCTCGATgtacgtctccgtccccatcttcacgtataccaacacccacacttc
      A  G  I  Q  L  L  L  D
561  ttccctacgatagggtacccccgcacgccttacgacgggatggacgaaccattgatctagctttcttctt
631  ggggactggactttgcaaagccaaactacctcggtagatcttcagttggccatgaaaagatggatgtgtt
701  ggaggaagggggaggggtgtttgatgctgctgcggtgggattcgacatgacatgatatgacagactggct
771  gacactgctgcgtgggtgttactatatagGCTGAGAGGGAAGCTACCAAGATTGTTCAGAAGgtatgttc
                                    A  E  R  E  A  T  K  I  V  Q  K
841  tcctttccccattgcattgtaattgtcgatgatgcatgcgtcggaaagaaacgtacgtcttgatataata
911  gttgctaacgcttggtatgatggttcgggggtatagGCTAGAGAGgtacgtattgcatcccagaaaccaa
                                        A  R  E
981  cgacctcacccgatatacccgataccctacccaagatcactcgacgttcctgcgccatcgacgtaaatg
1051 aggtcctgtcgatggggcaaatcattgtacaactgtaacaagaggccggaaacacggagacagcaggcag
1121 acggatgacctaccgtcacttatgacctagaccggcaaggtagtaggctaacgaaaacaaaacaaatgga
1191 acagTACCGCACAAAGCGCGTCCGCGAAGCGCGCGACGAAGCCAAGAAGGAGATAGAAGCGTACAAGGCA
        Y  R  T  K  R  V  R  E  A  R  D  E  A  K  K  E  I  E  A  Y  K  A
1261 CAAAAGGAGGCCGAGTTCAAGAAGTTTGAAGCCGAGgtatgtttcctccccttctgtcccttcttcctgc
     Q  K  E  A  E  F  K  K  F  E  A  E
1331 ttatcccccaccccttctccctctatcccccctgttttttttatttttcccacatccacaaaccatcaaca
1401 ggaaacaatgcaaggaaaacgtacatactaaacctacattatctgtacaataacagCACACACAAGGAAA
                                                          H  T  Q  G  N
1471 CCAAGCCGCCCAAGAGGAAGCCAACGCCGAAGCCGAGGCGCCGATTCGCGAGATCAAGGAGGCCGGCAAC
      Q  A  A  Q  E  E  A  N  A  E  A  E  A  R  I  R  E  I  K  E  A  G  N
1541 AAGAACCGCGAGCAGGTTATTAAGGATTTGTTGCATGCCGTGTTTACCCCTTCTCCTGAGGCCATGGCTG
     K  N  R  E  Q  V  I  K  D  L  L  H  A  V  F  T  P  S  P  E  A  M  A
1611 CTCACTAAGGGCAGTAACTGTACCTGGTTGGAACAAACAGGAATGGAAGGTGATGGGGCGACGATGGGGA
     A  H  *
1681 TGATGTTGGATTGGCGGATGATGTTGGATTGGCCTGGGCTGGCCCGTTATGGTAAACGACGAAAGGGGAG
                                                    Site of polyadenylation ↓
1751 GCCGCGCCGTTTTTCTGTCATGAAGGCATGTTAGGTTGTTAGACGGAGATTATGAAGTTGAGTTTGTGAT
1821 TTGGTATCACTATCTTACGGTTATGGTTACGTGGTTTGCGTTTAAGGTTCGAATTAGACCAGACCATGT
1891 CTCAAAGTACTATAGACAACTGGAATCGCCAAAATCCACAGTTTTCAATAACCAAAGGACAAGGTCTGTA
1961 AAGCCCGGACAGCGCAACCTGGTAGTATGAAAGAGCCTAGCCTAGCTCAATGGTATCTTTATGGATACTT
2031 GGTCATTACTTACTCACTCACTCACTCACTCACTCACTATTTCCCTTTTCTCAACCTATTCAACATGTCC
2101 TTCTACATCCCCTTGTACATCCCCCTTTTTCAGTCACGAAAAGAATACCTCAAAAACTCCGAACAGGCCT
2171 TCAACATCTCCCCCGTATCCCTCCCTTCCTCCGTCTCCGAATCCACCCCCGCCATCTGTCCCTCAACGTC
2241 CTCGCCAGCACTCCAAAGTCCTCACCCCGATCCTTCAACAATNTCCCTGATCTTTGCCCGACATCCCGTA
2311 TCTTCAGCAATCAACCATGCTAGTATCACGTCAATCGTCTGTAGGCAGAACAGTCCCGCGTCCAGTCTAC
2381 GAGAAAAGGCCTCGTCTTCGTCAAATTGTTCTGGACTGGAAGCTCCGTCCGCCTTGATGGCTTCGTCTAC
2451 TTGGCTGACGCCGCGGCGTACTCGCGACGAAGTCGAATGAGCTTGGAGGTCTTGGCGTATTCTTTTCTA
2521 CGAATTTGGCGAGGAGGCGAT
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Fig. 2. Sequence of the *vma-10* gene. The nucleotides which correspond to the begining and end of the cDNA are indicated with arrows. Introns are shown in lower case. Underlined regions of the derived amino acid sequence correspond to the sequence of peptides obtained from the 18-kDa polypeptide in the vacuolar ATPase.

tion of the intron found in the yeast *VMA10* gene (Supekova *et al.*, 1995). The conservation in position of this intron is intriguing, especially considering the rarity of introns in the yeast genome. The possible implications with regard to the evolution of this gene are discussed below.

Analysis of the 370 bps preceding the start codon suggests that *vma-10* shares several sequence features common to three other *vma* genes (*vma-1, vma-2*, and *vma-3*) which may be involved in regulation and expression. The first 100 bps of the upstream region are 70% G+C. 150 bp upstream of the start codon is a small region (30 bps) that is less than 40% G+C, the same pattern as observed in other *vma* genes (Wechser and Bowman, 1995).

Three conserved sequence motifs common to several *N. crassa* housekeeping genes are also apparent (Wechser and Bowman, 1995). The first sequence (GGGCG) can be found 360 bp from the start codon and the second (GGGCCG) is 200 bp away, while the third (CCAACTC) is 110 bp upstream and has been found within 30–60 bp of the major transcription site for three previously characterized *vma* genes *vma-1, vma-2*, and *vma-3*. Like housekeeping genes in animal and fungal cells, the *vma-10* gene does not appear to have a TATAAT box near the transcription start site.

The features shared by *vma-1, vma-2, vma-3*, and *vma-10* have not been found in all *vma* genes. Analysis of the 5′ untranscribed regions of *vma-4, vma-6*, and *vph-1* did not reveal any conserved sequence elements. It is noteworthy that the products of *vma-1, vma-2*, and *vma-3* (67, 57, and 16 kDa respectively) are all present in multiple copies in the ATPase (three copies of the 67- and 57-kDa subunits and six copies of the 16-kDa proteolipid per enzyme), while the gene products of *vma-4, vma-6*, and *vph-1* (26, 40, and 96 kDa respectively) are found in single copies (12). As shown in Fig. 1, preparations of the V_1 sector of the ATPase have an 18-kDa band that is more heavily stained than would be expected for a single-copy subunit. Reports from other organisms also suggest that there are multiple copies of the G subunit as discussed below. The conserved sequence elements in the 5′ untranscribed region could be involved in the expression of genes that encode subunits present in multiple copies in the enzyme.

Characteristics of the *vma-10* Protein

The *vma10* gene encodes a 115-residue protein with a molecular weight of 13,046 kDa and a PI of 7.24. The protein has no apparent membrane-spanning domain. It has a high proportion of charged amino acids, 19% arginine and lysine, 19% aspartate and glutamate, which may explain why this subunit appears to run as an 18-kDa protein on polyacrylamide gels. The homologous subunit in *S. cerevisiae* and *M. sexta* also migrates anomalously in polyacrylamide gels (Supekova *et al.*, 1995; Lepier *et al.*, 1996).

The *N. crassa vma-10* protein can be aligned with the three other *vma10* proteins that have been described (*S. cerevisiae, M. sexta*, and *B. tarus*) with 42%, 36%, and 31% identity respectively (Supekova *et al.*, 1995; Lepier *et al.*, 1996; Supekova *et al.*, 1996). As shown in Fig. 3 the most conserved part of the protein is the N-terminal half. Comparing the *N. crassa* and bovine proteins, 43% of the first 60 amino acids are identical. Almost half of the residues in this region are charged. By contrast the C-terminal half of the protein is poorly conserved: only 9 of the last 55 residues are identical when comparing the *N. crassa* and bovine proteins.

Although the sequence identity between G subunits in different organisms is not particularly high, the position of the conserved residues is very intriguing. When the N-terminal half of the polypeptide was modeled as an alpha helix essentially all of the conserved residues were positioned on the same face (Fig. 4). Comparing the *N. crassa, S. cerevisae*, and bovine polypeptides, one-third of the helix was nearly identical. These data strongly suggest that this region forms an alpha helix and that the conserved face interacts with another highly conserved region in the ATPase.

Comparison to the b Subunit in F-type ATPases

As first reported by Supekova *et al.* (1995, 1996) the N-terminal halves of the *vma-10* gene products are also similar in sequence to a region of the b subunit in the F-type ATPases. Alignment of the *N. crassa vma-10* protein with the b-subunits of *Vibrio alginolyticus* and *E. coli* showed 31% and 21% identity respectively. The alignment with the *E. coli* subunit is shown in Fig. 3. It should be noted that the b subunit is poorly conserved in F-type ATPases. For example, comparison of the bovine and *E. coli* subunits shows only 17% sequence identity. The striking difference between the b subunits of the F-type ATPases and the G subunits of the V-type ATPases is that the G subunit lacks a membrane-spanning domain. This probably explains the association of the G subunit with the V_1 sector, as opposed to association with V_0, as would have been

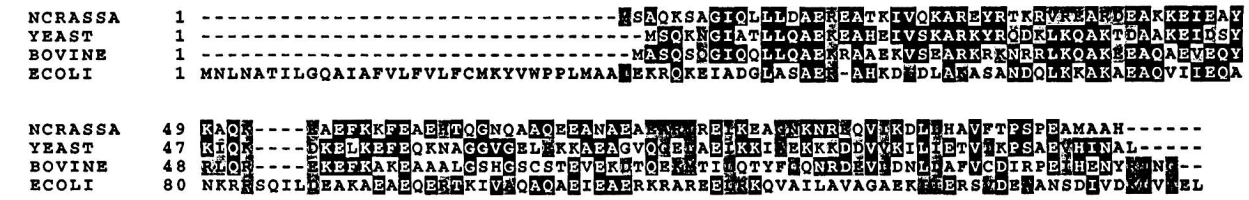

Fig. 3. Alignment of G subunits from vacuolar ATPases with the b subunit from *E. coli*. The sequences shown are from *N. crassa*, *S. cerevisiae* (Supekova *et al.*, 1995), *Bos taurus* (Supekova *et al.*, 1996), and *E. coli* (Walker *et al.*, 1984). Amino acids identical in two or more proteins are shown in dark boxes. Amino acids which are conservative substitutions are shown in shaded boxes.

predicted for the homolog of subunit b. In this regard it is intriguing to note that the position of the first intron in *S. cerevisiae* and *N. crassa* corresponds to the boundary between the hydrophobic and hydrophilic domains in the bacterial b subunit. The gene encoding the homolog of subunit b in the chloroplast genome (Cf_0 I) also has an intron at a similar position (Bird *et al.*, 1985). These data suggest that the G subunit and the hydrophilic region of the b subunit arose from a common ancestral protein which may have evolved separately from the membrane-spanning domain.

Because the G subunits and the b subunits have strong similarities and differences, it was important to see if homologous proteins were present in prokaryotic V-type ATPases. In the archaebacteria the primary ATP-driven proton pump appears to be more

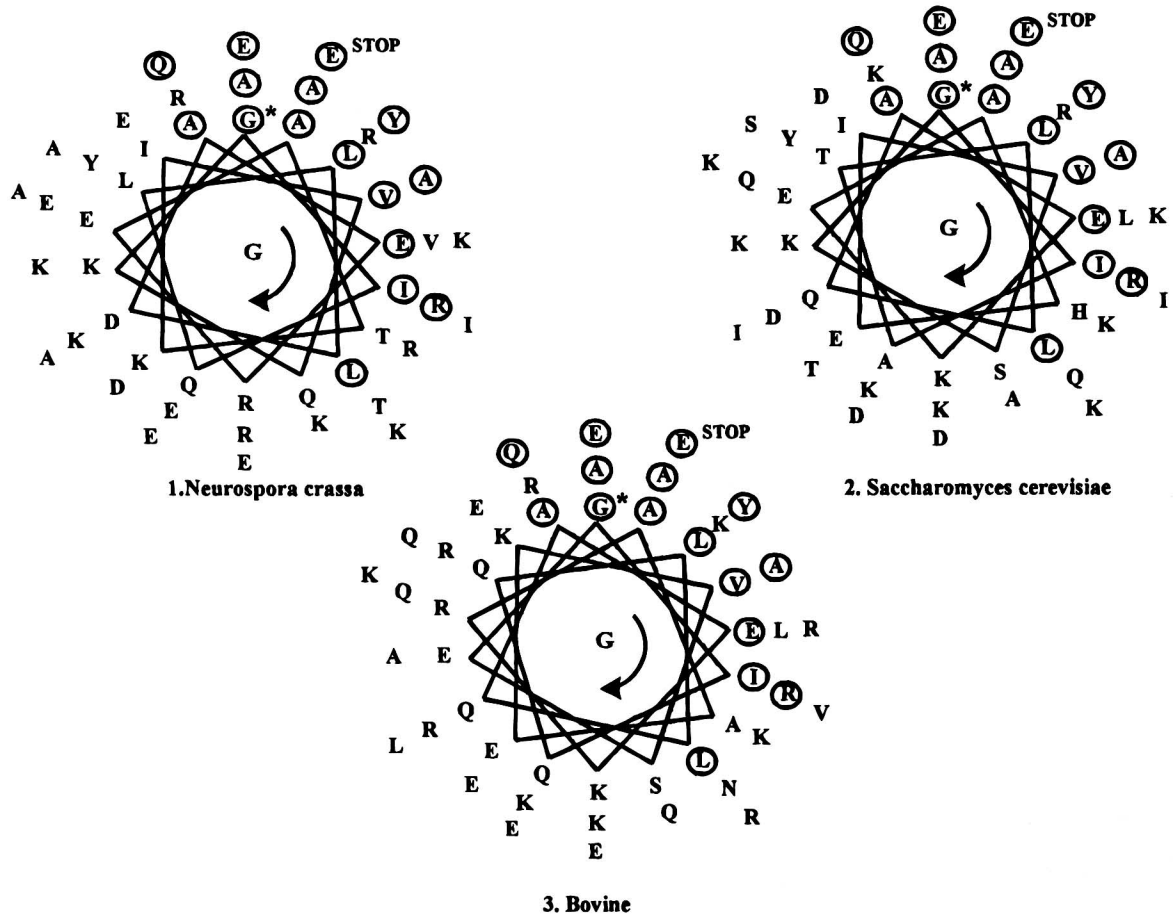

Fig. 4. Helical wheel diagrams of the N-terminal half of G subunits. Begining with the first conserved glycine residue (G8 in *N. crassa*, see Fig. 3) the position of 58 residues was plotted on a helical wheel diagram. The circled amino acids are identical in all three organisms.

closely related to V-type ATPases than to F-type ATPases. For example, analysis of the complete genome of *Methanococcus jannaschii* revealed no F-type ATPase, but did reveal an operon encoding at least seven subunits of a V-type ATPase (Bult *et al.*, 1996). We examined the genes flanking the reported operon and found that the protein encoded by the ML0223 gene is significantly similar in size and sequence to eukaryotic G subunits. As shown in Fig. 5, 33% of the first 45 residues in the polypeptide are identical in *M. jannaschii* and *N. crassa*. Like the b and G subunits 50% of the residues in the N-terminal half of the *M. jannaschii* protein are charged amino acids. A complete operon for a V-ATPase has also been identified in *Enterrococcus hirae* (Takase *et al.*, 1994). This organism is a eubacterium which contains both F-type and V-type ATPases (Takase *et al.*, 1993). Supekova *et al.* (1996) suggested that the *NtpG* gene in the *E. hirae* operon encodes the homolog of subunit G. However, in our analysis it appears that the product of the *NtpF* gene is more similar to the G and b subunits. The protein is of similar size, shows a significant degree of sequence identity in the N-terminal half, and has a high proportion of charged amino acids (see Fig. 4). Of further interest, in the operons from *M. jannaschii* and *E. hirae* all of the genes appear in the same order. The genes encoding the G subunit homolog is grouped with the two genes with encode integral membrane subunits, just as the gene encoding subunit b is grouped with the F_0 genes in the *E. coli* operon (Takase *et al.*, 1994; Bult *et al.*, 1996).

Stoichiometry and Membrane-Spanning Domains

The G subunit and the b subunit from both prokaryotes and eukaryotes appear to share a similar hydrophilic domain, but these subunits differ in two important aspects as summarized in Table I. First, the b subunit has a membrane anchor composed of apparently one hydrophobic helix in prokaryotic cells and two hydrophobic helices in mammalian cells (Walker *et al.*, 1982; Fillingame, 1990; Senior, 1990). The G subunits in V-type ATPases have lost this hydrophobic region. It is conceivable that another subunit has this function in V-type ATPases, but in the *M. jannaschii* and *E. hirae* operons only two genes appear to encode integral membrane proteins, and neither is likely to play the role of the b subunit (Takase *et al.*, 1994; Bult *et al.*, 1996).

Second, the number of G or b subunits in an individual ATPase may be different in different organisms. For the F-type ATPases the stoichiometry appears to maintain the number of membrane-spanning helices. For example, in *E. coli* each enzyme contains two copies of subunit b, each of which has one membrane-spanning helix. In *Rhodospirillum rubrum, Synechocystis*, and in chloroplasts each enzyme contains two different subunits called b and b′, each with one membrane-spanning helix (Bird *et al.*, 1985; Falk and Walker, 1988; Lill and Nelson, 1991). In bovine cells the enzyme contains only one b subunit, but that subunit has two membrane-spanning helices (Walker *et al.*, 1982; Collinson *et al.*, 1996).

The number of G subunits in a V-type ATPase has not been resolved. As shown in Fig. 1 the band at the position of the G subunit in *N. crassa* appears to stain more heavily than expected if only a single copy were present. Analysis of the V-ATPase in both *S. cerevisiae* and bovine cells indicated that each enzyme may contain three G subunits (Supekova *et al.*, 1995; Crider *et al.*, 1997). The data are difficult to interpret because non-identical G subunits may be present. Crider *et al.* have isolated two genes in bovine cells which encode G subunits that are 64% identical (Crider *et al.*, 1997). These could be tissue-specific isoforms, or they could be equivalent to the b and b′ subunits in some F-type ATPases. Crider *et al.* reconstituted the hydrophilic sector of the V-ATPase and found that addition of three copies of either of the G subunits yielded maximal ATPase activity.

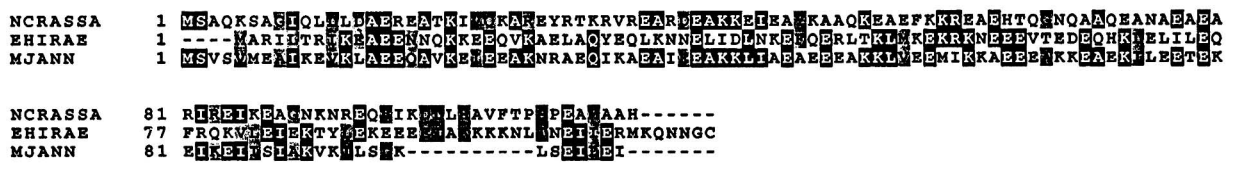

Fig. 5. Alignment of subunit G from *N. crassa* with homologous subunits from vacuolar ATPases in prokaryotic cells. The sequences shown are from *N. crassa, E. hirae* (Takase *et al.*, 1994), and *M. jannaschii* (Bult *et al.*, 1996). Amino acids identical in two or more proteins are shown in dark boxes. Amino acids which are conservative substitutions are shown in shaded boxes.

Table I. b and G Subunits in F-type and V-type ATPases

	Molecular weight (kDa)	Number of copies	Number of membrane helices
	F-ATPase		
Procaryotes			
E. coli subunit b	17	2	1
R. rubrum subunit b	19	1	1
R. rubrum subunit b′	17	1	1
Chloroplasts			
Spinach CFol	19	1	1
Spinach CFoll	16	1	1
Eucaryotes			
B. taurus subunit b	24	1	2
	V-ATPase		
Procaryotes			
M. jannaschii (0223)	12	?	0
E. hirae (NtpF)	14	?	0
Eucaryotes			
N. crassa subunit G	13	?	0
B. taurus subunit G	13	3	0
S. cerevisiae subunit G	13	3	0
M. sexta subunit G	13	?	0

In *N. crassa* we have preliminary data to indicate that the band at the position of the G subunit contains several different proteins. As described in Methods nine tryptic peptides were sequenced. Four of them were encoded by the *vma-10* gene and are thus derived from subunit G. Three other peptides were found to be encoded by a cDNA sequenced in the Neurospora Genome Project (Genbank # AF001033). This polypeptide has a calculated molecular mass of 19,125 Da, but has very little sequence similarity to the *vma-10* gene product. (Hunt and Bowman, unpublished results). Two other peptides were also obtained from the 18-kDa band, but the gene or genes encoding them have not yet been identified. It should be noted that the b and b′ subunits of the F-type ATPase in *R. spheroides* and the CFoI and CFoII subunits of F-type ATPase in chloroplasts share less than 20% sequence identity (Walker *et al.*, 1982; Bird *et al.*, 1985). Therefore, it will be important to determine if the other polypeptides in the 18-kDa band are part of the V-ATPase.

Possible Function of the b and G Subunits

Although the amino acid sequence of the b and G subunits is not particularly conserved, they appear to be homologous polypeptides in F-type and V-type ATPases, found in both prokaryotes and eukaryotes. In the F-type ATPase the b subunit is anchored in the membrane and also makes contact with the top of the F1 sector (Wilkens *et al.*, 1997). It has been suggested that the role of the b subunit is to serve as a stator that fixes the nucleotide-binding subunits to the membrane (Wilkens *et al.*, 1994; Cross and Duncan, 1996; Pedersen, 1996). If the G subunit plays the same role in the V-type ATPase, it is surprising that the polypeptide has no membrane anchor. The reason for this difference in the b and the G subunits may lie in the observation that the V-type ATPase can be regulated *in vivo*, by the reversible dissociation of the V_1 sector from the membrane (Kane, 1995; Sumner *et al.*, 1995). Subunit G may indeed bind to the membrane but by noncovalent interactions with other membrane subunits. In *N. crassa* and in *M. sexta* (Lepier *et al.*, 1996) subunit G appears to be part of the V_1 sector that dissociates from the membrane. If there are three G subunits per enzyme, as suggested by the data from experiments with the *S. cerevisiae* and bovine V-type ATPases, then each may be associated with an A and B subunit. In the vacuolar ATPases subunit G might serve to attach the nucleotide binding subunits to the membrane, but in a reversible manner.

We have shown that the G subunit may fold into an alpha helix in which one face is highly conserved.

This region could contact the A and B subunits which are also highly conserved. If this region is important with regard to subunit interactions then mutation of conserved residues in the G subunit may cause partial or full uncoupling of ATP hydrolysis and proton transport. We are presently undertaking mutagenesis experiments in an effort to examine this hypothesis.

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