The vacuolar type proton pump of clathrin-coated vesicles has a multisubunit ATP hydrolytic center that is peripheral to the membrane. Polypeptides present in this domain include the well characterized subunits A, B, C, D, E, and F; SFD, a dimer composed of 50- and 57-kDa polypeptides; and polypeptides termed G and H. Of these, subunits A, B, C, and E have been shown to be necessary but not sufficient for significant ATPase activity; in addition, either polypeptide G or H is also required for ATP hydrolysis (Xie, X.-S. (1996) J. Biol. Chem. 271, 30980–30985).

In this study, the polypeptides G and H were purified and directly sequenced. Subsequent molecular analysis has revealed that these proteins are isoforms, which we designate G1 and G2. The cDNAs encoding the rat and bovine brain and chicken osteoclast forms of G1 have been cloned. The open reading frames of these isoforms differ at only five residues; bovine G1 has 36% identity with VMA10, a component of the proton channel of yeast. Northern blot analysis revealed a 1.0-kilobase pair transcript encoding G1 in bovine brain, kidney, heart, and spleen. The cDNA encoding bovine polypeptide H was cloned and sequenced, revealing this protein to be 64% identical to G1, constituting isoform G2. In Northern blot analysis, a single 1.7-kilobase pair transcript hybridized with a probe to G2 in brain, but not in heart, kidney, or spleen. An antibody against a bovine G1-specific domain reacts with V pump from bovine brain, kidney, and chromaffin granule, whereas an G1-specific domain reacts with V pump from bovine heart, kidney, or spleen. An antibody against a bovine script hybridized with a probe to G2 in brain, but not in Northern blot analysis, a single 1-kilobase pair transcript encoding G1 in bovine brain, kidney, and chromaffin granule, whereas an anti-G2 antibody reacts only with proton pump from brain. The bovine forms of G1 and G2 were subsequently expressed in Escherichia coli and SF9 cells, respectively, and purified to homogeneity. Reconstitution of ATP hydrolysis was achieved by combination of recombinant subunits A, B, C, and E with either recombinant G1 or G2, demonstrating the role of these isoforms in pump function.

Vaccular, or V-type, proton pumps are remarkable for their wide distribution within eukaryotic cells, where they are found in most organelles, as well as for their intense phylogenetic conservation. Physiologically, these pumps are responsible for urinary acidification and bone resorption and participate in the respiratory burst phenomenon of macrophages. At a constitutive level, V-type proton pumps are essential for glycosylation in Golgi membranes, degradation of cellular debris in lysosomes, and processing of endocytosed receptor-ligand complexes in endosomes. In regulated secretory pathways, these proton-translocating ATPases energize neurotransmitter and catecholamine storage and facilitate processing of insulin as well as packaging of histamine and heparin (1–6).

Much is now known about the general structure of V-pumps. In all instances, they are multisubunit hetero-oligomers with a large peripheral sector, which catalyzes ATP hydrolysis, and a transmembranous proton channel (1–6). Investigation of the structure and function of the V-pump of clathrin-coated vesicles has revealed that the peripheral sector is composed of polypeptides of 70, 58, 40, 33, and 14 kDa, termed subunits A, B, C, E, and F, respectively (7–11). Recent work has also identified other components of this sector. These include a dimer composed of 57- and 50-kDa polypeptides termed SFD (sub-fifty-eight kDa dimer) (12), subunit D (13), and two polypeptides that we have designated G and H (14).

Definition of these components of the catalytic sector has been approached in several ways. It has been shown that the peripheral components of V-type pumps can be released from the proton channel by incubation with chaotropic agents, and/or nucleotides at 4 °C (15–16). These released polypeptides have been termed V₁, in analogy to the peripheral sector of the mitochondrial ATPase, which was termed F₁, (for Factor 1) (1). There has been no report of ATPase activity associated with these released subunits, and thus V₁ constitutes a group of peripheral subunits that are hypothesized to catalyze ATP hydrolysis. This suppression is well supported by nucleotide labeling experiments and identification of signature sites for ATP binding/hydrolysis in two of these peripheral components (subunits A and B) (17–21).

By another approach, we have sought to define by a functional analysis the elements responsible for ATP hydrolysis in V-type pumps. This has required dissociating the enzyme, isolating the separated subunits and/or subcomplexes, and determining by add-back experiments the requirement for ATP hydrolysis. Dissociation of the enzyme by several biochemical procedures (e.g. urea treatment) results in a change in the characteristics of the ATPase activity of the pump; namely, the enzyme loses its capability of hydrolyzing MgATP and switches to a form capable of only CaATP hydrolysis (22). We have termed this peripheral catalytic sector V₁, to distinguish these functional (and possibly structural) features from those of V₁.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
Recent studies have emerged that the catalytic sector of V pumps exists as a multisubunit subcomplex, has Ca\(^{2+}\)-activated ATPase activity, and reversibly associates with the transmembranous sector to increase pump number under physiological stimuli (23).

Our goal of defining the components of the pump responsible for ATP hydrolysis has centered upon identification of the minimal polypeptide requirements for CaATPase activity. This has resulted in the findings that four subunits (A, B, C, and E) are necessary, but not sufficient, for significant ATPase activity (7–10). Recently, it was found that an additional subunit was also necessary for ATPase activity and that either polypeptide G or H could fulfill this need. These studies were performed with recombinant subunits A, B, C, and E and biochemically prepared G and H polypeptides, and they were further remarkable for the fact that both CaATPase and MgATPase activity were reconstituted by these components (14).

To further explore the roles of these polypeptides in proton pump function, the molecular identities of polypeptides G and H have been investigated. Direct peptide sequencing, and cloning of their cDNAs reveal these components be highly homologous to each other and also to subunit G of the V pumps of yeast (24), *Manduca sexta* (23), and bovine chromaffin granule (25). These isoforms, which we term G1 and G2, differ in their tissue distributions. Northern blot analysis reveals that G2 is present in brain but not in kidney, liver, or spleen, whereas G1 is present in all tissues examined. G1 and G2 have been expressed in *Escherichia coli* and Sf9 cells, respectively, and purified to homogeneity. Reconstitution of MgATPase and CaATPase activity was achieved with recombinant subunits G1, G2, A, B, C, and E. In these experiments, it was determined that either G1 or G2, but not both, are required for reconstitution of ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, T, DNA ligase, and a nick translation kit for DNA probe labeling were purchased from Boehringer Mannheim; the GeneAmp PCR reagent kit with *Thermus aquaticus* Taq DNA polymerase and DNA sequencing materials and reagents were from Perkin-Elmer; a bovine brain cDNA library and a rat brain cDNA library in *zZAPII*, their host *E. coli* cells BB4 and XL1-Blue, and helper phage R408 were from Stratagene; *E. coli* expression vector pET16b and its host cell BL21(DE3) were from Novagen; baculovirus expression vector pHV1932 and baculovirus (AcNPV) DNA were from Pharmingen; [\(\alpha\)\(^{32}\)P]dCTP, [\(\alpha\)\(^{33}\)P]ATP, and an ECL kit for Western blot analysis were from Amersham Corp.; nitrocellulose membranes for plaque lift were from Millipore Corp.; and chemicals for SDS-PAGE were from Bio-Rad. Bovine adrenal chromaffin granule membranes were the generous gift of Dr. Joseph Albanesi (University of Texas Southwestern Medical Center at Dallas). All other reagents were obtained from Sigma.

**Peptide Sequencing**—Polypeptides G and H were separated by SDS-PAGE (15%) acrylamide), and proteins were electrophoretically transferred to Immobilon P filters, from which both polypeptide bands were excised and digested with trypsin (26). Released peptides were separated by reverse-phase high pressure liquid chromatography using a 2.1 \times 150-mm RP300 column (Perkin-Elmer) and were subjected to automated Edman degradation with a model 477A amino acid sequencer (Applied Biosystems, Inc.) using the manufacturer's standard program and chemicals.

**Cloning of cDNAs Encoding Subunit G1 from Rat, Bovine, and Chicken Sources—*zZAPII* phage DNA from an amplified rat brain cDNA library was purified by standard methods (26) and used as a template for PCR, which was performed with 1 \(\mu\)g of purified *zZAPII* DNA and 20 pmol of two synthetic oligonucleotide primers: 5'-GAAA-GAACCAGGAGCTGAGG-3' and 5'-CGGAAATGTAGTCTCGGAG-GATGG-3'. The sequences of these primers were based on direct peptide sequences and a homologous human EST sequence 3'-HPF03 (EMBL accession number Z43645). A 183-bp PCR product was cloned into pCR-II vector using a commercial TA cloning kit (Invitrogen). After positive selection by restriction enzyme digestion, the insert was excised with EcoRI and purified by preparative agarose gel electrophoresis. The 183-bp PCR fragment was labeled with [\(\alpha\)\(^{32}\)P]dCTP by nick translation and used to screen a rat brain cDNA library in *zZAPII* that had been transfected into *E. coli* strain BB4. Plaques were transferred to nitrocellulose membranes by a double-lift procedure (27). These membranes were then prehybridized for at least 4 h at 60 °C in a solution containing 5 \(\times\) SSC, 5 \(\times\) Denhardt's solution, 0.1 mg/ml sheared salmon sperm DNA, and 0.1% SDS. Hybridization was performed at 42 °C overnight with the same solution plus labeled probe, which was added at a concentration of 5 \(\times\) 10\(^6\) cpm/ml of hybridization solution. Duplicate positive clones were rescreened through one or more cycles until purified plaques were obtained.

Subunit G1 cDNA was cloned from a bovine brain cDNA library in *zZAPII*, using the same conditions for screening the rat brain library, except for a different probe, which was a 685-bp insert of a clone (R3–1) encoding rat brain subunit G1.

cDNA encoding chicken subunit G1 was cloned from a *zZAPII* Express cDNA library prepared from chicken osteoclasts using procedures similar to those described for cloning the cDNAs encoding the rat and bovine forms of subunit G1. Specifically, 1 \(\mu\)g of *zZAPII* Express phase DNA was used as template, and two synthetic oligonucleotides (5'-ATGGCAGTCGCA/GAAGAGAGGCAG-3' and 5'-CGGTATTCGTCGTA/GAATGGATTCGGG-3') were used as primers for PCR amplification. The resulting 242-bp PCR product was sequenced and found to be homologous to the cDNA encoding the rat and bovine forms of subunit G1. Subsequently, probe labeling and screening were carried out as described for cloning of the rat brain cDNA, except *E. coli* strain XL1 Blue MRF\(^{\prime}\) was infected with the *zZAPII* Express lysate. Purified clones were in *vivo* excised into pbCR-CMV using helper phage ExAssist and *E. coli* strain XLOLR. Four positive cDNA clones were identified as chicken subunit G1. Only one clone (COG303) contained the putative start codon and a 28-bp open reading frame.

**Cloning of cDNA Encoding Subunit G2 from Bovine Brain—Two**

oligonucleotide primers, 5'-AAGAGGAGGAGTCCGA/GAATGGAAAGGAAGCTGTCTGGAGG-3' and 5'-CTGCACTCCCTTGATC/GCTGACCTTGCIC-3', were synthesized in accord with two of the peptide sequences. Deoxynosine (I) was used in the 3' position of some cDNAs with a degeneracy of one or more. A 183-bp ZAP phage DNA from amplified bovine brain cDNA library was purified by standard methods (27) and used as a template for polymerase chain reaction. A 283-bp PCR product was sequenced in pCR\(^{\prime}\)I vector. The insert was excised with *Bam*HI and EcoRV, purified by preparative agarose gel electrophoresis, and used to screen a bovine brain cDNA library by a double-lift procedure, as described above.

**Sequence Determining—DNA Sequencing**—Digestion of all positive clones were excised and cloned into pBluescript or pbCR-CMV, with helper phage R408. Plasmid DNA was prepared by alkaline lysis, and DNA sequencing reactions were carried out with the ABI PRISM\(^{\prime}\) dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase, and subjected to sequencing analysis using a model 377 ABI PRISM DNA sequencer. The positive clones were fully sequenced in both orientations using T7, T3 promoter sequences, and sequence-specific oligonucleotides as primers. DNA and protein data base searches were performed using a P/GENE-based program.

**Northern Blot Analysis—Poly(A)+ RNA** (2 \(\mu\)g) from designated bovine tissues was denatured and fractionated by 1% formaldehyde-agarose gel electrophoresis and transferred to a Zeta-Blot blotting membrane (Bio-Rad). After baking at 80 °C in a vacuum oven for 1 h, the membrane was prehybridized for 4 h at 50 °C in a solution consisting of 50% formaldehyde, 1.5 \(x\) saline/sodium phosphate/EDTA, 1% SDS, 0.5% nonfat dry milk, 0.5 mg/ml denatured salmon sperm DNA. The probes for subunits G1 and G2, and \(\beta\)-actin were labeled with \([\alpha\)P]dCTP by nick translation and added to the hybridization solution at a concentration of 1 \(\times\) 10\(^6\) cpm/ml of solution. Hybridization was then carried out at 50 °C overnight. The membrane was sequentially washed for 15 min at room temperature with 2 \(\times\) SSC and 0.1% SDS, 0.5 \(\times\) SSC and 0.1% SDS, and subsequently 0.1 \(\times\) SSC and 0.1% SDS. A final wash was carried out at 60 °C with 0.1 \(\times\) SSC and 0.1% SDS for 30 min, and ethanesulfonic acid; NTA, nitrolotriacetic acid; PAGE, polyacrylamide gel electrophoresis; SFD, sub-fifty-eight-kDa dimer, 50–57-kDa polypeptide for function in a vacuolar proton pump of clathrin-coated vesicles; IPTG, isopropyl-1-thio-\(\beta\)-galactopyranoside; V\(_c\), the basolateral, catalytic sector of V-type proton pumps; V\(_p\), the peripheral, catalytic sector of V-type proton pumps; EST, expressed sequence tag.

1 P. Andersen and L. Lundberg, unpublished data.
autoradiography was performed with an intensifier screen at −80 °C for 5 days. Expression and Purification of Subunit G2 in E. coli—The coding region for bovine subunit G2 was amplified by PCR using cloned cDNA BE33 as template and two synthetic oligonucleotides (5′-AGCCCAT- CATATTGCCGACTACGTGCAGGACG-3′ and 5′-GCAATAGGATGCTCA-GAGCTGCTCTATACCTTTATTGCGG-3′) as primers, which were designed to contain NdeI and BglII restriction sites and initiator and stop codons at the 5′-ends, respectively, to enable cloning into the bacterial expression vector pET16b. The amplification reaction was performed in a Gene Machine thermal cycler using the following conditions: 1 min at 94 °C and 5 min at 57 °C, for a total of 30 cycles. The PCR product was size-fractionated by agarose gel electrophoresis, from which a single 380-bp fragment was purified; the fragment was identical to the cDNA sequence as determined by direct DNA sequencing. Expression vector pET16bG1 was constructed by replacing the NdeI and BamHI fragment of pET16b with the PCR-amplified fragment, which had been digested with NdeI and BglII. For expression of subunit G, E. coli strain BL21 (DE3) was transformed with expression vector pET16bG1 and grown and induced with IPTG at 37 °C as described (28).

pET16bG1 encodes a fusion protein containing a histidine tag at the amino terminus of the subunit G1, potentially allowing the protein to be readily purified with Ni-NTA resin. However, the expressed subunit G1 was largely aggregated into insoluble form (inclusion bodies) in E. coli, and thus the following purification protocol was developed.

Step 1: Cell Lysis and Isolation of Inclusion Bodies—500 ml of IPTG-induced BL21 (DE3) cells harboring pET16bG1 were harvested by centrifugation at 4000 × g for 15 min at 4 °C and resuspended in 6 l of lysis buffer (50 mM Tris HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl) containing 0.2 mM phenylmethylsulfonyl fluoride and 0.2 mg/ml lysozyme. The mixture was kept on ice and stirred occasionally for 20 min. Subsequently, 300 μl of 20% sodium cholate was added, and the mixture was incubated at 37 °C and continually stirred with a glass rod for 5–10 min. When the lysate became viscous, 40 μl of DNase I (1 mg/ml) was added. The lysate was kept at room temperature until it was no longer viscous (30–40 min), and then it was centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 10 ml of denaturing buffer consisting of 6 M guanidinium HCl, 0.1 M NaH2PO4, and 0.5% Triton X-100, pH 8.0. After centrifugation at 12,000 × g for 15 min, the supernatant was used for Ni-NTA resin chromatography.

Step 2: Ni-NTA Resin Chromatography and Renaturation of Recombinant Subunit G1—The supernatant from step 1 was mixed with 1 ml of equilibrated Ni-NTA resin (Qiagen), and the mixture was shaken for 1 h at room temperature. After centrifugation, the Ni-NTA resin was loaded into a small column, which was then washed with 120 ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 15 mM imidazole, 10 mM Tris-HCl, pH 8.0) containing a continuous urea gradient from 6 to 3 M with a flow rate of 1 ml/min. The protein was eluted with 10 ml of wash buffer containing 3 mM urea and 200 mM imidazole, and 1-M fractions were collected and analyzed by SDS-PAGE and Coomassie blue staining.

Step 3: Glycerol Gradient Centrifugation—For further purification and refolding of recombinant subunit G1, selected fractions from Ni-NTA chromatography were loaded onto a 10–20% glycerol gradient, which was prepared in 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 3 mM dithiothreitol, and 1 mM urea. After centrifugation at 180,000 × g for 22 h at 4 °C, fractions were harvested from the upper fractions of the gradient, subjected to SDS-PAGE analysis, and used for reconstitution experiments.

Expression and Purification of Subunit G2 in Insect Sf9 Cells—The coding region for subunit G2 of H+–ATPase was amplified by PCR using cloned cDNA as template and two oligonucleotides (5′-AACAACACAT-ATGCCCAATCGTACATCCUGGTTA-3′ and 5′-GGAGTGGCATGCTACTGGT- TAGGTCCTATACCTTTATTGCGG-3′) and cloned into the vector pET16b. Bacterial expression vector pET16bG2 was constructed by replacing the NdeI–BamHI fragment of plasmid pET16b with the PCR product after digestion with the same restriction enzymes. pET16bG2 was digested with XhoI and BamHI. The resultant 0.5-kilobase pair fragment, which contained the entire coding region for subunit G2, was cloned into the XhoI and BamHI sites of vector pVL1392 to generate the baculovirus expression vector pVL1392-G2.

Spodoptera frugiperda (Sf9) cells were grown in monolayer or in suspension culture at 27 °C in either Grace’s or IPL-41 medium with 10% heat-inactivated fetal bovine serum plus 0.1% pluronic polyol F68. Cells were split 1:5 for propagation every 3–4 days. Recombinant baculovirus was generated by co-transfection of Sf9 cells by the Lipofectin method with purified vector pVL1392-G2 DNA and linearized AcNPA viral DNA (9). Positive viral clones were isolated by plaque assay and identified by their ability to direct the expression of subunit G2 protein, as determined by SDS-PAGE and Western blot analysis. For expression and production of recombinant protein, Sf9 cells (2 × 107 cells/ml medium) were infected with recombinant baculovirus and grown in suspension at 27 °C for 60–72 h. 500 ml of infected Sf9 cells were harvested by centrifugation at 4,000 × g for 15 min. The cells were then resuspended in 50 ml of lysis buffer consisting of 50 mM NaH2PO4, 300 mM NaCl, 0.25% Tween 20, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Complete™, Roche Diagnostics) and sonicated three times for 1 min on ice. The lysate was then centrifuged at 40,000 × g for 1 h at 4 °C with a Beckman Ti 70 rotor. The supernatant was discarded, and the pellet was resuspended in 10 ml of denaturing buffer, consisting of 6 M guanidinium HCl, 0.1 M NaH2PO4, and 10 mM Tris-HCl, pH 8.0. After centrifugation at 15,000 × g for 15 min, recombinant subunit G2 was purified by Ni-NTA resin chromatography and refolded, using procedures similar to those described for recombinant subunit G1.

Reconstitution and Measurement of ATPase Activity—All of these components used for ATPase reconstitution are described herein and elsewhere (8–10). ATPase activities were measured by the liberation of 32P from [γ-32P]ATP. The assay solution consisted of 30 mM KCl, 50 mM Tris/MES (pH 7.0), 2.5 mM CaCl2 or MgCl2, and 2 mM [γ-32P]ATP (200–400 cpm/nmol). ATPase samples were preincubated with 2.5 μg of phosphatidylinerse for 2–5 min at room temperature, and reactions were initiated by the addition of 100 μl of assay solution. After incubation for 30 min at 37 °C, the reactions were terminated by the addition of 1 ml of 1.25 M perchloric acid, and the liberated 32P was extracted and counted as described (29).

Antibody Preparation and Western Blot Analysis—Two peptides, CEQRRQGQG-QQQRRQNRDE (specific to subunit G1) and CEQATRRQVQGMQQSQQRNRRDE (specific to subunit G2), were synthesized based on deduced protein sequence, coupled to keyhole limpet hemocyanin, and used for immunization of New Zealand White rabbits as described previously (30). For Western blot analysis, protein samples were separated by 15% SDS-PAGE and transferred electrothermally to nitrocellulose paper. Immunodetection was performed using immune serum at a 1:5,000 dilution and an Amersham ECL Western blotting system.

Miscellaneous Procedures—Bovine brain chlacrin-coated vesicle H+–ATPase, its peripheral catalytic sector (Vc), and integral membrane proton channel (Vp) were purified as described (22, 31, 32). Briefly, Vp was prepared by treating purified pump with urea, followed by glycerol gradient centrifugation, and Vp was harvested from upper fractions of glycerol gradients used for purification of the intact proton pump. H+–ATPases from bovine kidney and chromaffin granule membranes were isolated as described for the pump of bovine brain chlacrin-coated vesicles (22) except for the omission of hydroxyapatite chromatography. Protein determination (33) and SDS-PAGE (34) were performed as reported.

RESULTS

Sequence of Polyptides G and H—Direct protein sequencing of polyptides G and H was performed as described under “Experimental Procedures.” Shown in Table I are the determined amino acid sequences of tryptic peptides derived from G and H.

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Amount (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAALGSHG</td>
<td>10</td>
</tr>
<tr>
<td>QARMGQQNLSAEVEQAT</td>
<td>12</td>
</tr>
<tr>
<td>QARMGQQNLSAEVEQATR</td>
<td>10</td>
</tr>
<tr>
<td>QQGMSQSSQQR</td>
<td>9</td>
</tr>
<tr>
<td>QQGMSQSSQQR</td>
<td>5</td>
</tr>
</tbody>
</table>

Table I Amino acid sequences of tryptic peptides of polyptides G and H

After purified polyptides G and H were treated with trypsin, peptides were separated by reversed phase high performance liquid chromatography and sequenced as described under “Experimental Procedures.”
FIG. 1. Sequences of cDNA clones encoding the G1 isoforms from rat brain (A) and chicken osteoclasts (B) and the G2 isoform from bovine brain (C) and their deduced amino acid sequences. The nucleotide sequences of rat cDNA clone (R3–1) (A), chicken cDNA clone (COG03) (B), and bovine brain cDNA clone (B17–2) (C) were determined by sequencing double-stranded DNA in both directions. The amino acid sequences obtained from direct peptide sequencing are aligned below the translated amino acid sequences.
Cloning of cDNAs Encoding Subunit G1—Data base analysis using the amino acid sequences of polypeptide G (Table I) identified several homologous human EST sequences. As the sequences directly obtained from peptide sequencing were not ideal for generation of oligonucleotide primers, we used flanking sequences within the EST clones to guide primer synthesis. As described under “Experimental Procedures,” this led to the isolation of the 183-bp PCR product that was used to screen a rat brain cDNA library (5 × 10⁸ individual bacteriophage) in αZAPRI. Three positive clones, designated R3–1, R7–1, and R7–2, were isolated; all three clones had identical sequences at overlapping regions (data not shown). Among them, clone R3–1 was found to contain the full coding region. The complete sequence contains 885 base pairs consisting of a 63-bp untranslated region at the 5'-end, a 265-bp noncoding region at the 3'-end, and a 357-bp open reading frame (Fig. 1A).

To clone the cDNA encoding bovine subunit G1, the entire rat cDNA fragment (R3–1) was used to screen 5 × 10⁸ phages of a bovine brain cDNA library. Three positive clones (B33–1, B42–1, and B43–1) were identified. Clones B42–1 and B43–1 contained only partial coding regions, and clone B33–1 contained the entire open reading frame. All positive clones had identical sequences at the overlapping region of both coding and noncoding regions. The sequence of clone B33–1 includes a 357-bp open reading frame and untranslated regions of 76 bp at the 5'-end and 542 bp at the 3'-end. While these studies were being performed, the cDNA sequence of bovine subunit G1 was published elsewhere (25), and the sequences of our clones were identical to that published.

To clone the chicken G1, a 342-bp cDNA fragment was used to screen 6 × 10⁸ phages of a chicken osteoclast cDNA library in αZAP Express. Four positive clones (COG01–04) were isolated. All clones had identical sequences differing only in the length of their 5'-end (data not shown). Clone COG03 was found to contain the full coding region. The sequence of clone COG03 from the chicken osteoclast cDNA library is shown in Fig. 1C. The sequence of clone COG03 included a 357-bp open reading frame and untranslated regions of 28 bp at the 5'-end and 563 bp at the 3'-end.

Cloning of cDNAs Encoding Subunit G2—The primary amino acid sequences of polypeptide H (Table I) were used to design primers instrumental in developing a probe, as described under “Experimental Procedures.” After screening 5 × 10⁸ individual bacteriophages, nine positive clones, designated B3–1, B9–1, B11–1, B13–1, B15–1, B16–2, and B17–2 were isolated. DNA sequencing demonstrated that clones B3–1, B9–1, B13–1, B14–1, B15–1, and B17–2 contained full coding regions, and all of the positives had identical sequences at the overlapping region of both coding and noncoding regions, with the exception of clone B3–1, which had a 19-bp insertion within the coding region. (Since this would induce a frame shift, this was probably due to a library artifact.). The full sequence of bovine clone B17–2, which includes a 357-bp open reading frame and an untranslated region of 51 bp at the 5'-end and 955 bp at the 3'-end, is shown in Fig. 1B.

Analysis of the Deduced Amino Acid Sequences—The translations of open reading frames of the bovine, rat, and chicken subunit G1 clones predict 118-amino acid protein sequences with calculated molecular masses of 13,682, 13,710, and 13,552 Da, respectively, which is in close agreement with the apparent molecular mass of the bovine subunit G1 as assessed by SDS-PAGE. Such is also the case with bovine G2, where translation of the open reading frame of its clone predicts a 118-amino acid protein with a calculated molecular mass of 13,565 Da. Although G1 and G2 are nearly identical in mass, they have isoelectric points of 8.22 and 10.89, respectively, which likely accounts for observed differences in their mobilities during SDS-PAGE (14). Kyte-Doolittle analysis (35) predicted no membrane-spanning domains for G1 or G2. This characteristic is consistent with the experimental observation in our laboratory that subunits G1 and G2 copurify with the peripheral catalytic sector (VC) of the V-type ATPase of brain. The primary structures of the bovine, rat, and chicken subunits G1 are highly conserved. Only five amino acid residues were found to be different among the 118 residues of the rat and bovine forms, and only 18 amino acid residues were different in the bovine and chicken forms. Data base searches revealed that the predicted sequence of bovine subunit G1 shares 36, 53, and 100% identity to VMA10 (24), to the G subunits of the V pum of tobacco hornworm (23), and to the bovine chromaffin granule (25), respectively. Bovine subunits G1 and G2 share 64% identity. Bovine G2 has 34 and 51% homology with VMA10 and the G subunit of tobacco hornworm. In general, residues of the amino-terminal half of the protein are more highly conserved than those of the carboxyl-terminal region (Fig. 2).

Expression of Subunit G1 in E. coli and Purification of Recombinant Protein—The open reading frame encoding bovine G1 subunit was amplified by PCR and cloned into the bacterial expression vector pET16b, resulting in vector pET16bG1, as described under “Experimental Procedures.” E. coli BL21(DE3) cells harboring the vector were grown to A₆₀₀ = 0.7–0.9 at 37 °C. After IPTG induction and lysis, a dominant protein band was visualized by SDS-PAGE and Western blot (Fig. 3). About 10% of the total protein in induced BL21(DE3) cells was recombinant protein (Fig. 3, lane 3), which strongly cross-reacted with an anti-G1 antibody (Fig. 3B). Recombinant subunit G1 was solubilized and purified as described under “Experimental Procedures” and is shown in Fig. 3, lane 4. It appears to have a higher molecular mass than the native subunit G1 because of an additional factor Xa and histidine residues.

Expression and Purification of Recombinant Subunit G2—Bacterial expression vector pET16bG2, containing the entire coding region for the subunit G2, was used to express subunit G2 in E. coli. However, the resultant recombinant protein in E. coli was subject to severe proteolysis during the expression. Repeated attempts to control the proteolysis were unsucces-
ful. As an alternative, we constructed and purified a recombinant baculovirus containing the cDNA sequence encoding subunit G2 that was utilized to transfect insect Sf9 cells. The recombinant baculovirus directed the expression of a fusion protein containing a histidine tag at the amino terminus. As shown in Fig. 4, subunit G2 was expressed in Sf9 cells. Production of recombinant protein reached a maximal level in Sf9 cells after infection for 72 h, constituting approximately 3–5% of the total cellular proteins.

Although significant expression of the subunit G2 was obtained in Sf9 cells, the recombinant protein was largely aggregated, requiring the use of a guanidium-denaturing procedure as described under “Experimental Procedures.” The purified protein is shown in Fig. 4, lane 4.

**Expression of Subunits G1 and G2 in Different Tissues**—The tissue distributions of mRNAs encoding bovine subunits G1 and G2 were investigated by Northern blot analysis, as shown in Fig. 5. A single transcript of 1.0 kilobase pair in bovine brain, heart, kidney, and lung hybridized with the G1 probe (Fig. 5B), whereas the G2 probe hybridized with a single mRNA band of 1.7 kilobase pairs in bovine brain, but not in heart, kidney, or lung (Fig. 5A). Analysis with β-actin probe revealed that the amount of mRNA used for Northern blot analysis was roughly equal for all tissues (Fig. 5C).

To further demonstrate the differential expression of subunits G1 and G2, Western blot analysis was performed as demonstrated in Fig. 6. Subunit G2 was detectable only in the V-ATPase isolated from bovine brain, and not in the ATPases from the kidney and chromaffin granule (Fig. 6A). Subunit G1 was present in all pump preparations (Fig. 6B).

**A Subunit G Isoform Is Required for Significant ATPase Activities**—We have demonstrated that four subunits, A, B, C, and E, are necessary, but not sufficient, for V-ATPase activity (7–10). To investigate the role of subunits G1 and G2 in ATP hydrolysis, recombinant components (shown in Fig. 7) were tested for the ability to support ATP hydrolysis. As demonstrated in Table II, recombinant A and B subunits supported minor ATP hydrolytic activity (10) that was not stimulated by the addition of the recombinant C and E subunits. However, the addition of subunit G1 to these four recombinant subunits resulted in significant Ca2+ - and Mg2+ -activated ATPase specific activities of 0.43 and 1.1 μmol of Pi·mg of protein−1·min−1,
Subunit G of V-ATPase

FIG. 6. **Western blot analysis of V-ATPases from different sources.** A, anti-G2 peptide antibody; B, anti-G1 peptide antibody. Lanes 1–3, purified V-ATPases from bovine brain, chromaffin granule, and kidney, respectively.

![Image](Image 133x488 to 223x581)

![Image](Image 313x435 to 558x729)

**FIG. 7.** **SDS-PAGE of recombinant subunits used for reconstitution of ATPase activities.** Proteins were stained with Coomassie Blue. Lanes 1 and 2, recombinant subunits A and B from Sf9 cells; lanes 3 and 4, recombinant subunits C and E from E. coli; lane 5, recombinant subunit G1 from E. coli; and lane 6, recombinant G2 from Sf9 cells.

![Image](Image 135x629 to 221x729)

**FIG. 8.** **Effects of various concentrations of recombinant subunits G1 and G2 on reconstitution of ATP hydrolysis.** CaATPase (circles) and MgATPase (squares) activities were measured by the liberation of $^{32}$P from $[^{32}$P] as described under “Experimental Procedures,” using 0.42, 0.36, 0.09, or 0.2 $\mu$g of recombinant A, B, C, and E and designated amounts of recombinant G1 (open symbols) or G2 (filled symbols).

![Image](Image 261x424 to 513x543)

**FIG. 9.** **SDS-PAGE and Western blot analyses of the purified proton pump and its subcomplexes prepared from bovine brain.** A, Coomassie Blue staining; B, Western blot analysis using anti-G2 peptide antibody; C, Western blot analysis using anti-G1 peptide antibody. Lane 1, proton pump; lane 2, purified proton channel (V$_a$); lane 3, purified V$_c$.

Subunit G Is a Component of the Peripheral Catalytic Sector—Subunits G1 and G2 lack membrane-spanning domains, as determined by Kyte-Doolittle analysis (35) of the deduced amino acid sequence, supporting the notion that they belong to the peripheral catalytic sector (V$_c$) of the H$^+$-ATPase. In contrast, subunit G (VMA10) of the yeast and bovine V-ATPases have been proposed to be membrane-associated (24, 25). To address this issue, we generated a purified transmembrane sector (Fig. 9, lane 2). SDS-PAGE and Western blot analysis demonstrated that subunits G1 and G2 copurify with the peripheral sector, and not with the transmembrane sector (Fig.

![Image](Image 235x488 to 558x729)

### TABLE II

**ATPase activities with recombinant subunits**

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Ca$^{2+}$-ATPase activity</th>
<th>Mg$^{2+}$-ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, B</td>
<td>0.05 nmol $\cdot$min$^{-1}$$\cdot$assay$^{-1}$</td>
<td>0.08 nmol $\cdot$min$^{-1}$$\cdot$assay$^{-1}$</td>
</tr>
<tr>
<td>C, E</td>
<td>0.01 nmol $\cdot$min$^{-1}$$\cdot$assay$^{-1}$</td>
<td>0.01 nmol $\cdot$min$^{-1}$$\cdot$assay$^{-1}$</td>
</tr>
<tr>
<td>G$_1$</td>
<td>0.04 nmol $\cdot$min$^{-1}$$\cdot$assay$^{-1}$</td>
<td>0.04 nmol $\cdot$min$^{-1}$$\cdot$assay$^{-1}$</td>
</tr>
<tr>
<td>G$_2$</td>
<td>0.04 nmol $\cdot$min$^{-1}$$\cdot$assay$^{-1}$</td>
<td>0.04 nmol $\cdot$min$^{-1}$$\cdot$assay$^{-1}$</td>
</tr>
</tbody>
</table>

respectively. Subunit G2 can replace subunit G1 to support significant Ca$^{2+}$- and Mg$^{2+}$-activated ATPase activities of 0.8 and 2.0 $\mu$mol of Pi mg$^{-1}$ protein$^{-1}$ min$^{-1}$; these specific activities are about two times higher than those achieved by the reconstitution with subunit G1. The differences in specific activities reconstituted with subunit G1 or G2 are further illustrated by the titration experiments of Fig. 8.

**DISCUSSION**

A definition of the components of V pumps has been approached by several strategies. The finding that subunit A could be labeled with nucleotides led to the view that this component was the site of ATP hydrolysis. Comparisons of V pump architecture with that of the mitochondrial ATP synthase led to the notion that a prominent peripheral structure of V pumps (V$_c$) was similar to F$_1$, of the mitochondrial ATPase and thus was the catalytic domain of the enzyme. Release of components of V$_c$ from the membrane led to the identification of a collection of polypeptides that lacked ATPase activities but
were considered to constitute the catalytic core of the enzyme.

In a different approach, we have sought to define the components of the catalytic sector by reconstituting ATPase activities from isolated subunits. Initially, this entailed attempts at purifying the peripheral subunits and reassembling them to restore ATPase activity. Because of difficulties in obtaining sufficient amounts of pure components by standard biochemical procedures, we have instead tried to assemble the catalytic center from recombinant subunits where purity and quantity are not problematic. By using hybrid particles, composed of recombinant subunits and biochemically prepared subcomplexes, we have shown that subunits A, B, C, and E are necessary for ATP hydrolysis (7–10). Recently, it was determined that these four recombinant subunits could not together support ATP hydrolysis; rather, either of the biochemically isolated 14- and 15-kDa polypeptides of Vc, designated polypeptides G and H, were shown to be necessary to activate ATPase activity (14).

In this study we have molecularly characterized polypeptides G and H and have determined that they encode two closely related polypeptides, which have homology to the G subunit of the proton pump of yeast (24), tobacco hornworm (23), and bovine chromaffin granule (25). Because of differences in tissue distribution, it appears that these proteins are isoforms, which we term G1 and G2. This notion is supported by the demonstration that recombinant G1 and G2 may be interchangeably used to reconstitute ATP hydrolysis. Further, the essential nature of subunit G and the finding that only the G1 isoform is detectable in several tissues together indicate that it is unlikely that both G1 and G2 are required for the in vivo function of all V-type proton pumps. At present, we are uncertain of the significance of the observed differences (Table II and Fig. 8) when G1 and G2 are used to reconstitute ATP-hydrolysis. The isolation of recombinant G1 and G2 subunits were achieved by different procedures, and it is possible that the differences observed simply occur because one isoform has been retarded and purified in a more active form. However, it is perhaps notable that similar reconstitution experiments performed with biochemically prepared polypeptides G (subunit G1) and H (subunit G2) also demonstrate reproducible differences in the reconstitution of ATPase activities, and it thus remains a possibility that in brain the isoforms interplay to produce differences in pump activities.

In addition to demonstration of isoform variability in subunit G, we have succeeded in the reconstitution of ATPase activity from entirely recombinant components, thereby providing a system to define subunit interactions within the catalytic sector.

Questions remain regarding the roles of the other peripheral components of the V pump. At present, it appears that subunit F, which is not essential for ATP hydrolysis (14), may serve to structurally link the catalytic sector to Vb6, the proton channel. SFD has likewise been shown to be essential for linking ATP hydrolysis to proton flow (12). Biochemical, structural, and functional data from this work clearly place subunit G within the catalytic center of the enzyme, in accord with studies performed with the V pump of tobacco hornworm (23) and in contrast to previous reports that this subunit is membrane-associated (24, 25). In this respect, it notable that the yeast phenotype resulting from the knock-out of VMA10 gene resembles that observed when other Vl (but not Vo) subunits are deleted. This would further suggest that subunit G is not the V pump equivalent of subunit b of Fo, as has been proposed (25).

Despite this, it is likely that subunit G, like subunit F, may sit at the interface of the catalytic and transmembranous sectors and that its release from membrane components may vary depending upon the protocol used. This could account for the finding that the isolated Vl preparation from tobacco hornworm has both the G subunit and Ca2+-activated ATPase activity, whereas other preparations of Vl are inert. It will be of interest to determine where subunit G localizes by other Vl release protocols to investigate the possibility of Ca2+-activated ATPase activity in Vl preparations of other systems.

Ultimately, reconstitution of this catalytic center from purely recombinant components will allow for an understanding of the roles of subunit G and SFD in pump function and thereby define the entire catalytic requirements of the V pump of clathrin-coated vesicles.

REFERENCES

Subunit G of the Vacuolar Proton Pump: MOLECULAR CHARACTERIZATION AND FUNCTIONAL EXPRESSION
Bill P. Crider, Per Andersen, Allen E. White, Zhiming Zhou, Xinji Li, Jan P. Mattsson, Lennart Lundberg, David J. Keeling, Xiao-Song Xie, Dennis K. Stone and Sheng-Bin Peng

doi: 10.1074/jbc.272.16.10721

Access the most updated version of this article at http://www.jbc.org/content/272/16/10721

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 24 of which can be accessed free at http://www.jbc.org/content/272/16/10721.full.html#ref-list-1