Molecular Characterization of the 50- and 57-kDa Subunits of the Bovine Vacuolar Proton Pump*

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The vacuolar type proton-translocating ATPase of clathrin-coated vesicles is composed of two large domains: an extramembranous catalytic sector and a transmembranous proton channel. In addition, two polypeptides of 50 and 57 kDa have been found to copurify with the pump. These proteins, termed SFD (sub-fifty-eight-kDa dimer) activate ATPase activity of the enzyme and couple ATPase activity to proton flow (Xie, X.-S., Crider, B.P., Ma, Y.-M., and Stone, D. K. (1994) J. Biol. Chem. 269, 25809–25815). It has also been reported that the clathrin-coated vesicle proton pump contains AP50, a 50-kDa component of the AP-2 complex responsible for the assembly of clathrin-coated pits, and that AP50 is essential for function of the proton pump (Liu, Q., Feng, Y., and Forgac, M. (1994) J. Biol. Chem. 269, 31592–31597). We demonstrate through the use of anti-AP50 antibody, identical to that of the latter study, that hydroxylapatite chromatography removes AP50 from impure proton pump preparations and that purified proton pump, devoid of AP50, is fully functional.

To determine the true molecular identity of SFD, both the 50- and 57-kDa polypeptides were directly sequenced. A polymerase chain reaction-based strategy was used to screen a bovine brain cDNA library, yielding independent full-length clones (SFD-4A and SFD-21); these were identical in their open reading frames and encoded a protein with a predicted mass of 54,187 Da. The SFD-21 clone was then used in a reverse transcription-polymerase chain reaction-based strategy to isolate a related, but distinct, transcript present in bovine brain mRNA. The nucleotide and predicted amino acid sequences of this isolate are identical to SFD-21 except that the isolate contains a 54-base pair insert in the open reading frame, resulting in a protein with a predicted mass of 55,933 Da. Both clones had 16% identity to VMA13 of Saccharomyces cerevisiae. No sequence homology between the SFD clones and AP50 was detectable. Anti-peptide antibodies were generated against an epitope common to the two proteins and to the unique 18-amino acid insert of the larger protein. The former reacted with both components of native SFD, whereas the latter reacted only with the 57-kDa component. We term the 57- and 50-kDa polypeptides SFDα and SFDβ, respectively.

Vacular, or V-type, proton translocating ATPases are found in diverse eukaryotic endomembrane compartments and plasma membranes of epithelia, macrophages, and specialized polarized cells. Their functional roles reflect this broad distribution and range from processing receptor-ligand complexes in endosomes to osteoclast-mediated bone dissolution (1–3).

V-type proton pumps are complex hetero-oligomers with peripheral and intramembranous domains. Preparations of the proton pump of clathrin-coated vesicles contain at least nine proteins of 116, 70, 58, 57, 50, 40, 39, 34, and 33 kDa, as well as four smaller polypeptides with apparent molecular masses of 10–17 kDa (4–7). This polypeptide composition is similar to that of most V-type pumps, reflecting intense phylogenetic conservation (8–12). Functional domains of the coated vesicle proton pump include Vc, a proton channel that is probably composed of the 116-, 39-, 17-kDa components (5), and Vc, the catalytic sector of the complex consisting of a set of polypeptides of 70, 58, 40, 34, 33, 15, 14, and 10 kDa (6). Separation of Vc from the holoenzyme results in marked changes in enzyme activity; although native enzyme hydrolyzes Mg-ATP at a rate 3-fold higher than Ca-ATP, isolated Vc can hydrolyze ATP in the presence of Ca2+, but not Mg2+, and cannot support ATP-driven, vectorial proton movement (7). Recently, we reassembled purified VR and Vc to yield a complex with Mg-ATPase and proton pumping activities similar to those of native enzyme. Essential to this reconstitution was a heretofore unrecognized 50–57-kDa polypeptide heterodimer, which we termed SFD, for sub-fifty-eight-kDa dimer (6).

Others recently reported that the 50-kDa subunit of the AP-2 clathrin assembly complex is present in their preparation of the clathrin-coated vesicle proton pump and that AP50 might function as a kinase, catalyzing autophosphorylation as well as phosphorylation of the 58-kDa subunit of the pump (13). Subsequently, it was reported that AP50 was essential for the function and assembly of the clathrin-coated vesicle proton pump (14). Although such an interaction could explain the basis whereby endomembrane acidification is regulated by an organellar specific mechanism, these reports are not easily reconciled with the prior demonstrations that AP-2 (and its component, AP50) is found only in plasma membrane-associated clathrin-coated pits and vesicles (15, 16) and that clathrin-coated vesicles associated with the plasma membrane cannot

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) AF041337 and AF041338.

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1 The abbreviations used are: Vc, the bafilomycin-sensitive proton channel of V-type proton pumps; Vc, the peripheral, catalytic sector of V-type proton pumps; bp, base pair; C1, 20 poloxylene 9-lauryl ether; HTP, hydroxylapatite; PAGE, polyacrylamide gel electrophoresis; SFD (sub-fifty-eight-kDa dimer), the 50–57-kDa polypeptide heterodimer required for function of the vacuolar proton pump of clathrin-coated vesicles; PCR, polymerase chain reaction; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
acidify their interiors (17, 18).

To investigate this point, and to identify the 50- and 57-kDa polypeptides of SFD and their role(s) in pump function, we studied the possible relationship of the 50-kDa component of SFD to AP50. Recently, we reported that phosphorylation of 50- and 58-kDa polypeptides was observed only with impure clathrin-coated vesicle pump preparations and that purified pump required SFD for function but lacked any kinase activity (6). This observation, however, did not exclude the possibility that the 50-kDa component of SFD is AP50, since the primary structure of the latter (19) lacks any known nucleotide binding motif, and its role as a kinase is conjectural. We now demonstrate that AP50 itself is not a component of the purified clathrin-coated vesicle pump; nor is it required for ATP-driven proton translocation. Through direct protein sequencing and molecular cloning, we show that the 50- and 57-kDa components of SFD are highly related proteins that probably arise through an alternative splicing mechanism. These subunits are termed SFDα (57 kDa) and SFDβ (50 kDa). They share 16% identity to a 54-kDa polypeptide of the yeast vacuolar proton pump encoded by the VMA13 gene (20). No homology, however, exists between these proteins and AP50 or any AP isoforms. These studies demonstrate that SFD, and not AP50, is required for function of the vacuolar-type proton pump of clathrin-coated vesicles.

EXPERIMENTAL PROCEDURES

Preparations and Materials—Isolation of clathrin-coated vesicles from bovine brains (21) and preparations of liposomes from purified lipids (22) were performed as described. Purification of the proton-translocating ATPase of clathrin-coated vesicles was performed by sequential solubilization with C₆H₅OH, hydroxylapatite (HTP) chromatography, (NH₄)₂SO₄ fractionation, and glycerol gradient centrifugation (4); purified H⁺-ATPase had a specific activity of 14–16 μmol Pigen protein−1 min−1. Purified proton pump was depleted of SFD by treatment with Zwittergent 3-16, followed by glycerol gradient centrifugation, as reported (6). Purified AP-2 (μ2) complex from rat brain and chicken anti-AP50 (μ2) IgG were the generous gifts of Dr. Thomas Kirchhausen (Harvard University). Goat anti-chicken IgG antibody was obtained from Pierce, HTP (Bio-GEL HTP) was from Bio-Rad; restriction enzymes, T₄ DNA sequencing ligase, and a nick translation kit were from Boehringer Mannheim; DNA sequencing reagents and enzymes and the GeneAmp PCR reagent kit were from Perkin-Elmer; Escherichia coli strains XL1-Blue and BB4 and helper phages R408 were from Stratagene; radioactive reagents were from Amersham Corp.; nitrocellulose membranes for plaque lift were from Millipore Corp.; and chemicals for SDS-PAGE were from Bio-Rad. A bovine brain cDNA library was the generous gift of Dr. Richard Dixon, University of Texas Health Science Center at Houston. The sources of other materials used in this study have been identified previously (3–7).

Protein Sequencing—About 200 pmol of each of the 50- and 57-kDa components of SFD were separated by SDS-PAGE (12% acrylamide), and proteins were electropheretically transferred to Immobilon polyvinylidene difluoride filters, from which the 50- and 57-kDa polypeptide bands were excised, and digested with trypsin or LysC in situ (23). Released peptides were separated by reverse phase high performance liquid chromatography using a 2.1 × 150-mm RP300 column from Perkin-Elmer and were subjected to automated Edman degradation using a model 477A amino acid sequencer from Applied Biosystems with the manufacturer’s standard program and chemicals.

Synthesis of a DNA Probe by PCR—To obtain a DNA probe for screening a bovine cDNA library, four rounds of PCR were performed. The first PCR was carried out with a bovine cDNA library as template. Two primers (I, 5'-GGCGCCTGGT/C/TGC/TGC/TG/TGC/TC-3'; II, 5'-GTIAATGAIAGA/G/A/CT/TTG/CGIAGAIA-3') based upon primary peptide sequence. After 30 cycles of PCR, this reaction generated one dominant band of ~500 bp, but sequencing revealed that this arose from primer I annealing to two separate sites in a transcript that did not encode amino acids flanking those used to design primer I. Under the premise that a genuine, relevant PCR product had also been produced (at a much lower copy number than the ~500-bp product), a second PCR reaction was performed. The reaction mix was diluted 100-fold, and 1 μl of the diluted reaction mixture was used as template in a second PCR reaction. Primer II and primer III (III, 5'-TATCTCCCA/AG/GT/A/G/TTACCATIA/G/A/G/T/CT/TT/A-3') was designed according to an expressed sequence tag clone (TO6491) identified by screening expressed sequence tag data bases with peptide sequences obtained from the direct sequencing of the 50-kDa component of SFD. This second round of PCR amplified a 120-bp fragment. To generate a larger DNA fragment suitable for cDNA library screening, the blunt end of the 120-bp fragment was used to design two additional primers (IV, 5'-AGACTGGGTTGTCAACGAGATAA; V, 5'-CTTCC- GTACAGCCAGGGGGTGTGTT-3'). Primer IV and T3 promoter primer were used in the third round of PCR, using bovine brain cDNA library as template. The reaction mix was diluted 100-fold, and 1 μl of the diluted reaction mixture was used as template in the final round of PCR using primer V and T3 promoter primer. This round of PCR generated an 856-bp fragment that was used to screen the library.

Cloning of cDNAs Encoding SFD of Clathrin-coated Vesicle ATPase—The 856-bp PCR fragment was labeled with [α-32P]dCTP by nick translation and used to screen a bovine brain cDNA library in AZIP, which had been transfected into E. coli strain BB4. Plaques were transferred to nitrocellulose membranes by a double-lift procedure (26). These clones were then characterized for an ORF in a solution containing 5 × SSC, 5 × Denhardt’s solution, 0.1 mg/ml sheared salmon sperm DNA, and 0.1% SDS. Hybridization was performed at 60 in °C overnight with the same solution plus labeled probe, which was added at a concentration of 5–10 × 10⁹ cpm/ml of hybridization solution. Duplicate positive clones were re-screened through one or more cycles until purified plaques were obtained. About 1.3 × 10⁶ independent clones were screened, yielding two independent, full-length clones, SFD-4A and SFD-21.

Based on the sequences of SFD-4A and SFD-21, two primers were synthesized (VI, 5'-CTC TTCGCTGGT/C/TGC/TGC/TGC/TGC-3' and VII, 5'-GGCTGTCATCCATCCCCAGGTAGACT-3') and used for RT-PCR. In brief, 0.1 mg of bovine brain poly(A) RNA (24) was reverse transcribed using oligo(diT)ₙ as primer. PCR coding sequences were then amplified by PCR using primers VI and VII. Two fragments were amplified and were subsequently TA-cloned (24), using an In vitro TA cloning kit and the manufacturer’s protocol. Four full-length clones, SFD-RT1, -2, -3, and -4, were isolated from this approach.

Sequencing of SFD-RT1, -2, -3, and -4 was carried out with ABI PRISM™ 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 T₄ promoter, M13 reverse sequencing primers, and sequence-specific oligonucleotides as primers. DNA and protein data base searches were performed using PC GENE and the Wisconsin Sequence Analysis Package. Clones SFD-RT1, -2, and -3 were identical and were subsequently shown to encode the 50-kDa polypeptide of SFD, whereas clone SFD-RT4 encoded the related 57-kDa polypeptide of SFD, which we term SFDβ.

Antibody Preparation and Western Blot Analysis—A peptide common to the predicted translations of SFDα and SFDβ (CEMKRSPEEKQEM-LQTEGS) and an SFDα-specific peptide (CRLRBSGVTAEGTSSSSD) were synthesized, coupled to keyhole limpet hemocyanin, and used for immunization of New Zealand White rabbits (3), yielding Q48 and Q50 antisera, respectively. For Western blot analysis, protein samples were separated by 15% SDS-PAGE and transferred electrophoretically to nitrocellulose paper. Immunodetection was performed using immune serum at a 1:5,000 dilution and an Amersham ECL Western blotting system. Immunoblot protocols using rabbit polyclonal anti-70-kDa subunit antibody (1:5,000 dilution) have been described previously (3). Polyclonal anti-rat AP50 (μ2) IgG from chicken was used at a 1:20,000 dilution, and secondary antibody (goat, anti-chicken IgG) was used at a 1:40,000 dilution. Rabbit polyclonal anti-39-kDa antisera was generated against recombinant bovine brain 39-kDa polypeptide. This antisera was used at a 1:5,000 dilution. Protein determination (25) and SDS-PAGE (26) were performed as reported.

Assay of Proton Pumping—Partially purified proton pump of clathrin-coated vesicles was reconstituted into liposomes prepared from pure lipids by the freeze-thaw, cholate dilution method, as reported (23). Proton pump was assayed by ATP-dependent quenching of acridine orange in an Amino DW 2C dual wavelength spectrophotometer as ΔA₄92/540. Proteoliposomes (1.2 μg of protein) were diluted into 1.5 ml of assay buffer consisting of 150 mM KCl, 2.5 mM MgCl₂, 10 mM Na-Triicine (pH 7.0), and 7 μM acridine orange. Reactions were initiated by the
addition of 1.3 mM ATP and 1 μM valinomycin and were terminated with the addition of 1 μM bis-(hexafluoroacetonyl)acetone.

RESULTS

Fig. 1 illustrates key preparations used to investigate the possible role of AP50 in function of the clathrin-coated vesicle proton pump. SDS-PAGE (panel A) and immunoblot analysis (panel B) with an anti-AP50 antibody, were performed as described under “Experimental Procedures.” Lane 1, bovine brain clathrin-coated vesicles (3 μg); lane 2, H+ -ATPase, partially resolved by HTP chromatography (1.1 μg); lane 3, purified H+ -ATPase (2.4 μg); lane 4, purified SFD (0.18 μg); lane 5, purified μ2 complex (1 μg); lane 6, purified recombinant AP50 (0.2 μg).

FIG. 1. Identification of AP50 in clathrin-coated vesicle H+-ATPase preparations. SDS-PAGE, followed by Coomassie Blue staining (panel A) and Western blot analysis (panel B) with an anti-AP50 antibody, were performed as described under “Experimental Procedures.” Lane 1, bovine brain clathrin-coated vesicles (3 μg); lane 2, H+ -ATPase, partially resolved by HTP chromatography (1.1 μg); lane 3, purified H+ -ATPase (2.4 μg); lane 4, purified SFD (0.18 μg); lane 5, purified μ2 complex (1 μg); lane 6, purified recombinant AP50 (0.2 μg).

The results of Fig. 1 indicate that AP50 was not present in our pump preparation after HTP chromatography. To further address this point, the distribution of polypeptides eluted during HTP chromatography was examined by SDS-PAGE, as shown in Fig. 2A. Lanes 3–10 show fractions sequentially eluted by a linear (0–300 mM) NaPi gradient; lane 11 shows 20 μl of the HTP chromatography fraction eluted by 1 M NaPi step wash after completion of gradient.

FIG. 2. AP50 is removed from the clathrin-coated vesicle H+-ATPase by HTP chromatography. HTP chromatography, SDS-PAGE (panel A), and Western blot analysis with anti-AP50 antibody (panel B), anti-70-kDa antibody (panel C), and anti-39-kDa antibody (panel D) were performed as described under “Experimental Procedures.” Lane 1, purified H+ -ATPase (2.4 μg); lane 2, purified rat μ2 complex (1 μg); lanes 3–10, 20 μl of the HTP chromatography fractions eluted by 0 (lane 3) to 300 mM (lane 10) linear NaPi gradient; lane 11, 20 μl of the HTP chromatography fraction eluted by 1 M NaPi step wash after completion of gradient.

The 57- and 50-kDa subunits of SFD were treated with trypsin, eluted from polyvinylidene difluoride membranes, and subjected to high performance reverse phase liquid chromatography as described elsewhere (24) and under “Experimental Procedures.” Panel A, trypsin digest of the 57-kDa component of SFD; panel B, trypsin digest of the 50-kDa component of SFD. Peaks T1, T2, and T8 (panel A) and T2 and T8 (panel B) were subjected to Edman degradation; resulting sequences are listed in Table I.
a fraction eluted by a 1 M NaPi wash performed after the gradient. Also illustrated are Western blot analysis performed on the fractions of panel A, using antibodies directed against AP50 (panel B), the 70-kDa proton pump subunit (panel C), and the 39-kDa proton pump subunit (panel D). As shown (panel B), AP50 is not eluted by the standard gradient used to prepare proton pump (lanes 3–10) but was released by a 1 M NaPi step wash (lane 11). In contrast, proton pump components are effectively eluted by the 0–300 mM gradient, as indicated by the simultaneous elutions of the 70-kDa subunit of Vc (panel C) and the 39-kDa component of Vb (panel D) in lanes 3–7. Notably, neither peripheral (70-kDa) nor membrane-associated (39-kDa) components are eluted by the 1 M NaPi wash (panels C and D, lane 11), demonstrating full separation of the pump components and AP50.

Next, the HTP elution fraction shown in Fig. 2, lane 5, was subjected to glycerol gradient centrifugation. Resultant purified proton pump (Fig. 1A, lane 3) that is devoid of AP50 (Fig. 1B, lane 3) catalyzes a high rate of proton pumping after reconstitution into liposomes prepared from pure lipids (Fig. 3). Taken together, these data indicate that the 50-kDa component of the V-pump of clathrin-coated vesicles is not AP50, claims to the contrary (13, 14) notwithstanding.

To determine the genuine molecular identity of the 50- and 57-kDa components of SFD, these polypeptides were purified and subjected to Edman degradation. Both were blocked at their amino termini, and internal peptides were generated by digestion with trypsin or LysC. After separation by reverse phase high performance liquid chromatography, Edman degradation was performed as described under “Experimental Procedures.”

The first indication of similarity of the 57- and 50-kDa polypeptides came from the pattern of peptides generated by trypsin digestion. As shown in Fig. 4, nearly identical elution profiles were obtained for the peptides derived from the 57- and 50-kDa polypeptides (panels A and B, respectively). Shown in Table I are the primary sequences obtained from analysis of trypptic and LysC-derived peptides. Notably, sequences obtained from peaks (T2 and T8) of the 57- and 50-kDa polypeptides were identical. Comparison of these sequences with those of data bases revealed that there was no significant sequence homology except for T1, which showed identity with a human expressed sequence tag (T0649).

As outlined under “Experimental Procedures,” the primary sequences of the peptides prepared from the 50- and 57-kDa polypeptides were used to generate an 856-bp PCR product that was then used to probe a bovine brain cDNA library; ultimately, two independent, full-length clones (SFD4 and SDF21) were isolated. The sequence of SFD21, its predicted amino acid sequence, and alignment with the peptides of T1, T2, T8, T22, and Table I are underlined.

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TABLE I
Sequences of trypptic and Lys C peptides of the 57- and 50-kDa subunits of SFD

| Peptide Sequence (designation) Amount |
|--------------------------------------|
| T1 QLQSEQPOTAAR 7 |
| T2 SVIEQLQGGK 14 |
| T8 LGQQLQDSSSF 3 |
| T2 SGIVERQK 14 |
| T8 LGQQLQDSSSFADYS 3 |
| L22 LLEVSRDPQVLAVAHVDGYEVHRHPYV 10 |

SFD-21

| Peptide Sequence (designation) Amount |
|--------------------------------------|
| T1 QLQSEQPOTAAR 7 |
| T2 SVIEQLQGGK 14 |
| T8 LGQQLQDSSSF 3 |
| L22 LLEVSRDPQVLAVAHVDGYEVHRHPYV 10 |

TABLE I
Sequences of trypptic and Lys C peptides of the 57- and 50-kDa subunits of SFD

After purified 57- and 50-kDa polypeptides of SFD were treated with trypsin or LysC, peptides were separated by reversed phase high performance liquid chromatography and sequenced, as described under “Experimental Procedures.”

The first indication of similarity of the 57- and 50-kDa polypeptides came from the pattern of peptides generated by trypsin digestion. As shown in Fig. 4, nearly identical elution profiles were obtained for the peptides derived from the 57- and 50-kDa polypeptides (panels A and B, respectively). Shown in Table I are the primary sequences obtained from analysis of trypptic and LysC-derived peptides. Notably, sequences obtained from peaks (T2 and T8) of the 57- and 50-kDa polypeptides were identical. Comparison of these sequences with those of data bases revealed that there was no significant sequence homology except for T1, which showed identity with a human expressed sequence tag (T0649).

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Fig. 5. Nucleotide sequences of SFD-21 and SFD-RT4 and amino acid translations. The full nucleotide sequence of clone SFD-21 is shown; in boldface type is the unique 54-bp insert present in clone SFD-RT4. The locations of SFD-derived peptides T1, T2, T8, and L22 (listed in Table I) are underlined.
and L22 are shown in Fig. 5. The open reading frame encodes a polypeptide composed of 465 amino acids with a predicted molecular mass of 54,187 Da.

Because of the apparent homology found by direct sequencing of the 50- and 57-kDa subunits of SFD, we sought to clone a second cDNA that might encode the other of the two polypeptides. As outlined under “Experimental Procedures,” a PCR-based approach ultimately yielded four clones (SFD-RT1, -2, -3, and -4) from bovine brain poly(A)-enriched mRNA. Of these, SFD-RT1, -2, and -3 were identical to one another and to SFD21. SFD-RT4, however, had a sequence identical to SFD21, except that it contained a 54-bp insert, resulting in a protein composed of 483 amino acids with a predicted molecular mass of 55,933 Da. The nucleotide sequence of SFD-RT4 is compared with that of SFD21 in Fig. 5. As shown, these sequences are identical in both the coding and noncoding regions, save the 54-bp insert at nucleotide position 727 of SFD21. The predicted amino acid alignments of SFD-RT4, and SFD21 are of course identical, except for the putative 18-amino acid insert at position 175 (Fig. 6). Based on the differences in mass of the predicted proteins encoded by SFD-RT4 and SFD21, we presumed that these were equivalent to the 57- and 50-kDa components of SFD, respectively. Provisionally, we assume that these two proteins are isoforms, and we term the product of SFD-RT4 (and the 57-kDa polypeptide) SFD\textsubscript{a} and the product of SFD-21 (and the 50-kDa polypeptide) SFD\textsubscript{b}.3 Data bank

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3 The predicted masses of SFD\textsubscript{a} and SFD\textsubscript{b} are in approximate accord with molecular radii based upon electrophoretic mobility. At present, we have no evidence of posttranslational modification of either species.
searches revealed that SFD polypeptides have 53% (gene Ti4F91.1) and 40% (gene F52E11.10) identity to probable homologues in Caenorhabditis elegans, 23% identity to a probable homologue (gene SPAC7D4.10) in Schizosaccharomyces pombe, and 16% identity to a 54-kDa subunit of the vacuolar proton pump of S. cerevisiae that is encoded by the VMA13 gene (20); alignments of the predicted amino acid sequences of these proteins with SFDα and SFDβ are shown in Fig. 6.

To further establish the relationship of these two polypeptides to SFD, we generated antibodies to synthetic peptides predicted from the translated nucleotide sequences of the 57- and 50-kDa components of SFD. The first of these, Q48, was generated against an epitope common to SFDα and -β, while the second, Q50, was generated against the predicted unique 18-amino acid insert present in SFDα. Shown in Fig. 7 are the results of Western blot analysis using these antibodies. As shown, antisera directed against the common epitope (Q48) reacts with both the 57- and 50-kDa subunits present in SFDα. In contrast, antibody Q50, directed against the unique 18-amino acid insert of SFDα, reacts only with the 57-kDa component of purified pump (panel C, lane 1) and SFD (panel C, lane 3). Neither antisera reacted with V-pump that had been selectively depleted of SFD (panels B and C, lane 2).

DISCUSSION

The results of this study demonstrate that the clathrin-coated vesicle proton pump is highly active in the absence of AP50 (Fig. 3) and that SFDα and -β are distinct from AP50. It is important to note that these conclusions are in part based on the use of the identical antibody used in the previous reports (13), indicating that AP50 was required for function of the clathrin-coated vesicle proton pump. We believe differences between our results and those published previously are in part due to variations in the methods of preparation of active H^+-ATPase from clathrin-coated vesicles that were employed in the two studies.

Purification of the clathrin-coated vesicle proton pump, in our hands, is achieved by an extended protocol consisting of solubilization of stripped clathrin-coated vesicle membranes with C_{12}E_{6}g followed by centrifugation at 150,000 × g for 60 min, HTP chromatography, (NH_4)_2SO_4 fractionation, and glycerol gradient centrifugation. Enzyme, thus prepared, has a specific activity of 14–16 μmol P_i·mg protein·min^{-1} and is dependent upon phospholipid (optimally, phosphatidyserine) for activation of ATPase activity, and liposomes for reconstitution of proton pump activity (4). In contrast, reports of the role of AP50 in the function of clathrin-coated vesicle proton pump were based on studies of enzyme prepared by an abbreviated version of our protocol. The isolation protocol of these studies (13, 14) consisted of treating stripped vesicles with C_{12}E_{6}g, followed by centrifugation at 100,000 × g, and glycerol gradient centrifugation. The resultant preparation has a specific activity of 6 μmol P_i·mg protein·min^{-1}. It is thus possible that the preparation of proton pump used by others was contaminated with AP50 and that their attempts to directly sequence the 50-kDa component yielded residues from the amino terminus of AP50, but not SFDβ, which was not susceptible to amino terminus sequencing in our hands. Other claims regarding the role of AP50 in pump structure and function, such as the reported stoichiometry of about 1 mol of AP50/mol of proton pump (13, 14) remain inexplicable. In particular, the report that the AP2 complex is itself fundamentally required for both pump activity and the reassembly of dissociated proton pump components (14) is in direct conflict with the previous biochemical (6) and current molecular characterizations of SFD from our laboratory. Irrespective of these points, it is clear that inclusion of HTP chromatography as a pump purification step results in separation of AP50 from fully functional enzyme (see above); thus, we find no support for the notion (14) that AP50 is required for acidification catalyzed by the proton pump of clathrin-coated vesicles.

Molecular cloning of the components of SFDα and -β demonstrates that they differ only by a 54-hp “insert” present in the former. Because of nucleotide identity at all other positions, including noncoding regions at the 3′- and 5′-ends of the clones, it is most likely that this difference in sequences is due to alternative splicing, as has been shown for a number of V-pump subunits. It is further possible that SFDα and SFDβ are isoforms. Biochemical studies will be required to determine if there are requirements for both components in the function of a single pump or whether a single form is sufficient for function.

Sequence homology of SFDα and -β with the VMA13 product (20) underscores the apparent similarity in function of these components in V-pumps from bovine brain and yeast, respectively. Specifically, SFD was shown to activate Mg-ATPase activity of the V-pump of clathrin-coated vesicles and to functionally couple ATP hydrolysis to proton flow. Also, V-pump selectively depleted of SFD remains intact but cannot support proton pumping (6). These characteristics are highly similar to those observed in VMA13 knock-out experiments, where it was found that the V-pump of yeast vacuoles, although assembled, was inactive with regard to ATPase and proton pumping activities (20).

Site-specific regulation of V-pump activity is an issue of broad interest that relates to the maintenance and generation of different pH gradients in organelles of the constitutive trafficking pathways and to the role that this interorganelle pH gradient plays in targeting nascent proteins to their ultimate cellular destinations. Although it is possible that AP50 and V-type proton pumps interact to produce some currently undefined function, our data refute the notion that AP50 is an organelle-specific regulator of V-pump function and specifically demonstrate that AP50 is not required for pump activity. We thus are focused on determining the molecular characteristics of SFD and its role in the function of V-type proton pumps.

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