Identification of a 14-kDa Subunit Associated with the Catalytic Sector of Clathrin-coated Vesicle H\(^+\)-ATPase*

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The clathrin-coated vesicle H\(^+\)-ATPase is composed of a peripheral catalytic sector (V\(_c\)) and an integral membrane proton channel (V\(_b\)), both of which are multiple subunit complexes. This study was conducted to determine if subunit F, previously identified in vacuolar proton pumps of tobacco hornworm and yeast, was present in mammalian pumps. Using a polymerase chain reaction-based strategy, we have isolated and sequenced cDNA clones from bovine and rat brain cDNA libraries. A full-length clone from rat brain encodes a 119-amino acid polypeptide with a predicted molecular mass of 13,370 Da and with approximately 72 and 49% identity to subunit F of tobacco hornworm and yeast, respectively. Southern and Northern blot analyses indicate that the protein is encoded by a single gene. An anti-peptide antibody, directed against deduced protein sequence, was affinity-purified and shown to react with a 14-kDa polypeptide that is present in a highly purified pump prepared from clathrin-coated vesicles and also isolated V\(_c\). When stripped clathrin-coated vacuolar and purified chromaffin granule membranes were treated with KI in the presence of ATP, the 14-kDa subunit was released from both membranes, further indicating that it is part of the peripheral catalytic sector. In addition, direct sequencing of this 14-kDa component of the coated vacuolar proton pump confirmed its identity as a subunit F homologue.

Vacular proton pumps are responsible for the acidification of numerous cellular compartments, including clathrin-coated vesicles, endosomes, lysosomes, and Golgi membranes. In addition, these pumps acidify cellular vacuolar of the regulated secretory pathway, where they are instrumental to the packaging and processing of the contents of synaptic vesicles, mast cell granules, and insulin granules of pancreatic islet beta cells. Global loss of vacuolar pump function confers a conditionally lethal phenotype in Saccharomyces cerevisiae cells, while organ-specific loss of pump activity can result in renal tubular acidosis. In contrast, relative pump lethal phenotype in Chinese hamster ovary and pancreatic islet beta cells.

Close examination of preparations of active V\(_c\) reveals several additional proteins with molecular masses in the range of 10–15 kDa (17). Recently, a subunit of similar mass has been identified in vacuolar-type pumps of tobacco hornworm (18) and yeast (19). In these systems, evidence has been presented that this component (subunit F) is required for pump function and that it may serve to link the catalytic sector to the proton channel. However, it has also been shown that antibodies directed against this 14-kDa subunit of tobacco hornworm do not cross-react with any component of the clathrin-coated vesicle proton pump (18).

Experimental Procedures

Cloning of the cDNA Encoding the 14-kDa Subunit—Two oligonucleotide primers, GGTATGCTTGATGGGAATG/CGA(GA)GA(T/C)AC and GCCGGC(TTG)GGGAGAGTG/TGGTG(A/G)ATC(T/G)TT, were designed in accord with two conserved peptide sequences of the 14-kDa subunit of vacuolar ATPase from Manduca sexta (18) and yeast (19). Deoxyxynosine (I) was used in the third position of the indicated codons with a degeneracy of 2 or more. λZAP II phage DNA from amplified bovine and rat brain cDNA libraries was purified by standard methods (20) and used as a template.

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¶The abbreviations used are: V\(_b\), the bafilomycin-sensitive proton channel of the clathrin-coated vesicle proton pump; V\(_c\), the dissociated catalytic domain of the clathrin-coated vesicle proton pump, which hydrolyzes Ca\(_2\)ATP; PAGE, polyacrylamide gel electrophoresis.
for polymerase chain reaction performed with 40 pmol of each primer and 1 μg of purified XZAPII DNA. A polymerase chain reaction product of 272 base pairs was purified, labeled with [α-32P]dCTP by nick translation, and used to screen bovine and rat brain cDNA libraries in XZAPII that had been transfected into Escherichia coli strain BB4. Plaques were transferred to membranes by a double-lift procedure. The membrane (nitrocellulose) was then prehybridized for at least 4 h at 60 °C 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 42 °C for 12 h with the same solution plus labeled probe. Double-positive clones were rescreened through one or more cycles until purified plaques were obtained (20).

Inase from positive clones were excised and subcloned into pBluescript with helper phage R408. Plasmid DNA was prepared by alkaline lysis, and DNA sequencing was carried out by theideoxy termination method (21) using double-stranded and/or single-stranded DNA as a template. Single-stranded DNA was recovered from pBluescript in the presence of helper phage VCSM13. The cDNA clones were fully sequenced in both orientations using T7 and T3 promoter sequences and template. Single-stranded DNA was recovered from pBluescript in the presence of helper phage VCSM13. The cDNA clones were fully sequenced in both orientations using T7 and T3 promoter sequences and sequence-specific oligonucleotides as primers. DNA and protein data base searches were performed using PC/GENE-based programs.

Northern Blot Analysis—Poly(A)+ RNA (2 μg) from bovine tissues and total RNA (20 μg) from rat tissues were denatured and fractionated by 1% formaldehyde-agarose gel electrophoresis and transferred to Zeta-Probe membrane (Bio-Rad). After baking at 80 °C for 1 h on a vacuum oven for 1 h, the membranes were prehybridized for at least 4 h at 50 °C in a solution containing 50% formamide, 1.5 × saline/sodium phosphate/EDTA, 1% SDS, 0.5% nonfat dry milk, 0.5 mg/ml denatured salmon sperm DNA, and 1 μg/ml poly(A). The probes (the entire bovine and rat cDNA sequences, as shown in Figs. 1 and 2) were labeled with [32P]dCTP by random priming and were added to the hybridization buffer at a concentration of 1 × 106 cpm/ml of buffer. Hybridization was then carried out at 50 °C overnight. The membranes were sequentially washed for 15 min at room temperature with 2 × SSC and 0.1% SDS, 0.5 × SSC and 0.5% SDS, and 0.1 × SSC and 0.1% SDS. A final wash was carried out at 68 °C with 0.1 × SSC and 0.1% SDS for 30 min, and autoradiography was performed with an intensifier screen at −80 °C for 4–7 days.

Southern Blot Analysis—Genomic DNA was isolated from fresh bovine brain as described (20). Restriction enzyme digestions were performed on 100 μl of reaction solution with 10 μg of genomic DNA and 50 units of restriction enzymes at 37 °C for 12 h. Digest products were extracted and separated by 1% agarose gel electrophoresis and transferred to a Zeta-Probemembrane. Prehybridization, hybridization, and washing conditions were the same as those used for Northern blot analysis.

Preparation of Anti-14-kDa IgG and Western Blot Analysis—A synthetic peptide (CEIPSKEPHYDAKD) based on deduced protein sequence was coupled to keyhole limpet hemocyanin and used for immunization of New Zealand White rabbits as described previously (22). Anti-14-kDa IgG was affinity-purified from immune serum by affinity chromatography using a 2-ml Sulfolink® coupling gel column (Pierce) according to the manufacturer's instructions. Affinity-purified IgG was used for Western blot analysis, performed using an ECL immunoblot kit, as described (14). Preparation and use of anti-70-kDa antibody have been described elsewhere (15, 22). Rabbit polyclonal anti-39 kDa antisera was generated against recombinant bovine 39-kDa subunit.2

Release of the Catalytic Sector (VC) from Vacuolar ATPases—The peripheral membrane components of vacuolar ATPases were prepared by two methods. In the first method, proton-translocating ATPase was purified from clostridin-coated vesicles, and release of the active catalytic sector from purified ATPase was performed as reported (7, 8). The purification procedure (Fig. 2) is shown in Fig. 4 (lane 2).

In the second method, bovine brain clostridin-coated vacuoles, stripped of clathrin, and bovine clathrin co-clathrin membranes (generous gift of Dr. David Apps, University of Edinburgh) were suspended in 50 mM NaCl, 30 mM KCl, 20 mM HEPES (pH 7.0), 0.2 mM EDTA, and 5 mM ATP and incubated for 30 min on ice. Subsequently, 300 mM KI (final concentration for VC) was added to the mixture, and after 1-h incubation on ice, the mixtures were centrifuged for 1 h at 45,000 rpm in a Beckman Ti-60 rotor. Supernatants containing inactive peripheral components were concentrated by trichloroacetic acid precipitation and analyzed by SDS-PAGE (23) and Western blotting.

Peptide Sequencing—The subunits of purified H+-ATPase from clostridin-coated vacuoles were separated by SDS-PAGE (15% acrylam-
data banks revealed the predicted sequence of subunit F of *Drosophila melanogaster* and *Caenorhabditis elegans*; these are included in Fig. 3. Attempts to align the 14-kDa protein sequences with all known subunits of F1F0-ATPases revealed no significant homology.

A Single Gene Encodes the 14-kDa Subunit of the Clathrin-coated Vesicle ATPase—
Poly(A) RNA from bovine tissues and total RNA from rat tissues were hybridized with the designated inserts from the positive clones VIII-1 and E-1, respectively. In both species, only one message of ≈0.7 kilobase was observed in brain, heart, kidney, liver, lung, and spleen (Fig. 4). Southern blot analysis (Fig. 5), performed with the same DNA fragment used for Northern blot analysis, revealed only one dominant hybridization band, further indicating that the 14-kDa subunit is encoded by only one gene.

Identification of the 14-kDa Polypeptide as a Subunit of the Clathrin-coated Vesicle H⁺-ATPase—To determine the relationship of the cloned cDNA to components of the clathrin-coated vesicle H⁺-ATPase, we generated an anti-peptide antibody directed against an amino acid sequence predicted from the 14-kDa peptide clone. The antibody specifically cross-reacts with a 14-kDa polypeptide of highly purified clathrin-coated vesicle H⁺-ATPase (Fig. 6), indicating the presence of subunit F in the clathrin-coated vesicle proton pump. In addition, the 14-kDa polypeptide of the native enzyme was isolated by SDS-PAGE, eluted from preparative gels, and directly sequenced. An 11-residue sequence of NRHNPFLVEK was obtained. This sequence perfectly matched the deduced amino acid sequence of residues 22-33 of the rat (and bovine) clones, as shown in Fig. 1.

Association of the 14-kDa Subunit with the Peripheral Catalytic Sector of Vacuolar H⁺-ATPase—The 14-kDa peptide lacks membrane-spanning domains as determined by Kyte-Doolittle analysis of the deduced amino acid sequence, indicating the possibility that the 14-kDa protein belongs to the peripheral catalytic sector (VC) of the H⁺-ATPase. To investigate this point, we generated VC by treating the highly purified clathrin-coated vesicle ATPase with 3M urea. Isolated VC contains a polypeptide of appropriate mass that reacts with the 14-kDa specific antibody (Fig. 6B, lane 2). In addition, stripped clathrin-coated vesicles and chromaffin granule membranes were treated with KI in the presence of ATP as described under
**Fig. 7. Western blot analysis of the clathrin-coated vesicles (A) and chromaffin granule membranes (B) before and after digestion of peripheral pump components by treatment with KI and ATP.** Antibodies directed against the 70-, 39-, and 14-kDa subunits were used for immunoblotting as indicated. Lane 1, purified proton pump; lane 2, supernatants of vesicles incubated with KI and ATP; lane 3, control supernatants of vesicles incubated without KI and ATP as described under “Experimental Procedures.”

“Experimental Procedures.” This procedure causes the release of peripheral membrane-associated proteins from organelles, including components of vacuolar-type proton pumps, and has been used to define an inactive assortment of vacuolar pump components. These preparations, which are inactive, have been designated Vₜ by others (25, 26). As demonstrated by Western blot analysis (Fig. 7), the 14-kDa polypeptide and other peripheral pump subunits (e.g. subunit A) were released from membranes, whereas the 39-kDa polypeptide, a component of the proton channel Vₐ, was not, indicating that the 14-kDa subunit is part of the peripheral catalytic sector.

**DISCUSSION**

In the decade since a vacuolar-type proton pump was first isolated and reconstituted (7), considerable efforts have been directed toward defining the composition of these pumps as well as understanding the role of defined subunits in pump function. Investigations of these issues by the approach of resolution and reconstitution have led to the identification of two general sectors: a proton channel, Vₐ (12), and a catalytic domain, Vₜ (8). Both of these sectors, when separated from one another, have activities that are probably latent under physiological conditions, and this is likely of considerable importance. Vₐ, when purified and reconstituted, cannot conduct protons until it is activated by acidity (12). In a cellular context, this property may be essential to preservation of organelle pH gradients during the biogenesis (or regulation) of vacuolar-type pumps; specifically, a closed proton channel would prevent rapid proton leaks from acidic compartments. Likewise, the subunits responsible for ATP hydrolysis undergo a marked transition when released from Vₜ by select procedures. Namely, the isolated, functional, catalytic sector, termed Vₜ, can no longer hydrolyze MgATP, and it hydrolyzes CaATP only in the presence of millimolar concentrations of calcium. This property potentially prevents idle hydrolysis of ATP when the catalytic sector is not membrane-associated (8, 17).

We have utilized the partial reactions catalyzed by isolated Vₐ and Vₜ to define the components of each of these sectors and thereby the structure and function of the holoenzyme. In a series of studies, biochemically prepared Vₜ was selectively depleted of individual polypeptides, and these subunit-depleted Vₜ preparations were assessed for ATPase activity before and after readdition of the missing component. To assure purity of the latter, each of four subunits was cloned, expressed, purified, and renatured. Collectively, these experiments demonstrated that all four polypeptides of 70, 58, 40, and 33 kDa are subunits of Vₜ, and each is required for Ca-ATPase activity (13-16). Attempts to reassemble Ca-ATPase activity solely from these four recombinant subunits, however, have not been successful. As all of these subunits were shown to be active by reconstitution to subunit-depleted complexes, it appears that another component(s) is required for catalytic activity.

Potential candidates for such function(s) are several small polypeptides with molecular masses in the range of 10–15 kDa that are present in biochemical preparations of both the holoenzyme and Vₜ (Fig. 6A, lane 2). Close inspection of these components reveals the presence of three distinctive polypeptides within this mass range. Collectively, the experiments of this study identify one of these polypeptides as subunit F, thus demonstrating for the first time the presence of this component in vacuolar-type proton pumps of mammalian organelles. It is likely that the previous failure to identify this component by immunoblot analysis (18) owed to differences in the primary structures of subunit F of bovine and hornworm vacuolar-type pumps.

It remains to be determined what role this polypeptide plays in overall pump function. Studies conducted with the vacuolar pumps of tobacco hornworm and yeast indicate that subunit F may structurally couple the ATP hydrolytic sector to the proton channel (18, 19). Whether this entails an involvement in ATP hydrolysis per se remains to be determined, although inhibitory antibodies and gene knockout experiments indicate an essential role for subunit F in the net reaction of ATP-driven proton flow. Current experiments are directed toward the identification and characterization of the remaining two small polypeptides in Vₜ and toward ultimately defining the roles of these components and subunit F in overall pump function.

**REFERENCES**

1. Stone, D. K., and Xie, X.-S. (1988) Kidney Int. 34, 403–413
2. Alpern, R. A., Stone, D. K., and Rector, F. C. (1990) in *The Kidney* (Brenner, B. M., and Rector, F. C., eds) W. B. Saunders Co., Philadelphia, pp. 318–379
3. Forcà, M. (1989) Physiol. Rev. 69, 765–796
4. Mattsson, J. P., Schlesinger, P., Keeling, D. J., Teitelbaum, S. L., Stone, D. K., and Xie, X.-S. (1994) J. Biol. Chem. 269, 24979–24982
5. Al-Awqati, Q. (1986) Annu. Rev. Cell Biol. 2, 179–199
6. Marneli, M. H., Mathis, L. S., Stookey, M., Shia, S.-P., Stone, D. K., and Draper, R. K. (1994) J. Cell Biol. 125, 1907–1916
7. Xie, X.-S., and Stone, D. K. (1988) J. Biol. Chem. 262, 2492–2495
8. Xie, X.-S., and Stone, D. K. (1995) J. Biol. Chem. 270, 9859–9866
9. Denda, K., Konishi, J., Oshima, T., Date, T., and Yoshida, M. (1988) J. Biol. Chem. 263, 17251–17254
10. Kwon, P. M., Kuenz, M. C., Howard-Stevenson, I. H., and Stevens, T. H. (1992) J. Biol. Chem. 267, 447–454
11. Bowman, B. J., Allen, R., Wechsler, M. A., and Bowman, E. J. (1988) J. Biol. Chem. 263, 14002–14007
12. Crider, B. P., Xie, X.-S., and Stone, D. K. (1994) J. Biol. Chem. 269, 17379–17381
13. Peng, S.-B., Stone, D. K., and Xie, X.-S. (1993) J. Biol. Chem. 268, 23519–23523
14. Peng, S.-B., Zhang, Y., Tsai, S. J., Xie, X.-S., and Stone, D. K. (1994) J. Biol. Chem. 269, 11356–11360
15. Peng, S.-B., Zhang, Y., Crider, B. P., White, A. E., Fried, V. A., Stone, D. K., and Xie, X.-S. (1994) J. Biol. Chem. 269, 27778–27782
16. Peng, S.-B. (1995) J. Biol. Chem. 270, 16926–16931
17. Xie, X.-S., Crider, B. P., Ma, Y.-M., and Stone, D. K. (1994) J. Biol. Chem. 269, 25809–25813
18. Graf, R., Lepler, A., Harvey, W. R., and Weiczorek, H. (1994) J. Biol. Chem. 269, 3767–3774
19. Crider, B. P., Hill, K. J., and Stevens, T. H. (1994) J. Biol. Chem. 269, 25974–25977
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
22. Söudhof, T. C., Fried, V. A., Stone, D. K., J. oehston, P. A., and Xie, X.-S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6067–6071
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Fernandez, J., Day, M., Aaltonen, D., and Mische, S. M. (1992) Anal. Biochem. 201, 255–264
25. Moriyama, Y., and Nelson, N. (1989) J. Biol. Chem. 264, 3577–3582
26. Harvey, W. R. (1992) J. Exp. Biol. 172, 1–17
27. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132

3 X.-S. Xie, S.-B. Peng, B. P. Crider, and D. K. Stone, unpublished data.