Chapter 16
Eukaryotic V-ATPase and Its Supercomplexes: From Structure and Function to Disease and Drug Targeting

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Abstract The eukaryotic vacuolar-type ATPase (V-ATPase) is a multi-subunit membrane protein complex, which is evolutionarily conserved from yeast to human. It is also functionally conserved and operates as a rotary proton pumping nano-motor. In the first part of this chapter we discuss the structure and function of the yeast V-ATPase (V_1V_O) holoenzyme, We focus on the structural features of its sub-units forming both catalytic V_1 and proton conducting V_O sectors. Particularly, the recently solved structure of DF-subunit complex is discussed in relation to the energy coupling and regulation of yeast V-ATPase. It is noteworthy that the structure could contribute to understanding the function and regulation of V-ATPases of eukaryotes including human, leading to the rational design of specific inhibitors for medical applications. In addition to the well characterized role as proton pump, V-ATPases have acquired alternative cellular functions during evolution. In the second part we analyze novel roles of V-ATPase in function, signaling, and vesicular trafficking of cellular receptors. Our recent studies have uncovered that V-ATPase

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itself functions as an evolutionarily conserved pH-sensing and signaling receptor, which forms super-complex with aldolase/cytohesin-2/Arf1,6 small GTPases in early endosomes. On the other hand, V-ATPase forms a super-complex with mTORC1/Ragulator/Rag/Rheb small GTPases in late endosome/lysosomes and is involved in amino-acids sensing and monitoring nutritional state of cells. Finally, we discuss the role of V-ATPase in the development and progression of various diseases including cancer, diabetes, and osteopetrosis among others. We also present emerging approaches and future perspectives for specific drug targeting to V-ATPase and its super-complexes.

Keywords Eukaryotic V-ATPase • Holoenzyme • Acidification • pH-sensing - Receptor signaling • Super-complex • Vesicular trafficking • Human diseases - Drug targeting

Abbreviations

a2N	N-terminal cytosolic tail of a2-subunit V-ATPase
Arf1	ADP-ribosylation factor 1
Arf6	ADP-ribosylation factor 6
BafA1	*bafilomycin A1*
c/c″-ring	Ring composed by the c- and c″-subunits
ConA	*concanamycin A*
CRP	Calorie restriction pathway
cryo-EM	Cryo-Electron microscopy
CTH2	Cytohesin-2
dErbB	Dimeric EGFR/ErbB-receptor
EGF	Epidermal growth factor
EmGFP	Emerald green fluorescent protein
FKPB12	FK506/rapamycin binding protein
FRET	Fluorescence resonance energy transfer
Fz	Frizzled
GH	Growth hormone
HRG-1	Heme-responsive gene 1 protein
IGF-1R	Insulin-like growth factor-1 receptor
IR	Insulin receptor
LRP6	Low-density receptor-related protein
M. sexta	*Manduca sexta*
mErbB	Monomeric EGFR/ErbB-receptor
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
PAT1	Proton coupled amino acid transporter 1

PI3K	Phosphatidylinositol 3-kinase pathway
PKA	Protein kinase A
PPI	Protein–protein interaction interface inhibitors
RagA/C	Rag A/C GTPases
Ragulator	Ragulator complex
Ras	Rat sarcoma small GTPase
RAVE	Regulator of ATPase of vacuoles and endosomes
Rbcn-3	Rabconnectin-3A/B
Rheb GTPase	Ras homolog enriched in brain
S. cerevisiae	*Saccharomyces cerevisiae*
SAXS	Small-angle X-ray scattering
*Sc*DF1 and *Sc*DF2	Two conformations of subunit DF complex
TFEB	Transcription factor EB
TSC complex	Tuberous sclerosis complex
V-ATPase	V-type ATPase
ΔpH	Proton gradient
$\Delta\Psi$	Membrane potential

1   Introduction

Eukaryotic vacuolar-type ATPases (V-ATPases) are ATP-dependent proton pumps, which are localized in plasma membrane and the organelle membranes, and involved in various cellular processes [1–12]. This enzyme consists of a cytosolic V_1 and a membrane embedded V_O sectors. The subunit stoichiometry of the V_1 and V_O sectors are proposed to be $A_3{:}B_3{:}C_1{:}D_1{:}E_3{:}F_1{:}G_3{:}H_1$ and $a_1{:}d_1{:}c_x{:}c'_y{:}c''_z$, respectively [1–5]. Although yeast enzyme has only two isoforms (Vph1p and Stv1p) for a subunit, human and mice have multiple subunit isoforms including: (1) two for the B, E, H and d-subunits; (2) three for the C and G-subunits; and (3) four for a-subunit (a1, a2, a3, and a4). The expression and targeting of V-ATPase with these isoforms are specific for cells and organelles [1–5].

The hydrolysis of ATP into ADP and phosphate (Pi) in the A_3B_3 catalytic hexamer of V_1 sector drives the proton translocation by a ring of c, c', and c'' subunits of V_O sector. The coupling of both events is mediated through the rotation of a complex of DFdcc'c''. The reversible assembly/disassembly of the V_1 and V_O is a crucial mechanism for the regulation of V-ATPase [13–16]. Originally, this mechanism was discovered in *Manduca sexta* (*M. sexta*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) in response to ceased feeding [17, 18] and glucose depletion [13, 19], respectively. However, similar mechanism should be essential for mammalian V-ATPase [20–24].

V-ATPases play a central role in the maintenance of pH-homeostasis at the cellular and organism level in mammals [1–5]. This enzyme is also involved in the endosomal pH-sensing [1, 2, 25–30] and has most recently been uncovered as a

signaling receptor that modulates the activity of cytohesin-2 (CTH2) and Arf small GTPases [31]. In addition, V-ATPase is involved in sensing of amino acids and monitoring nutritional status of cells via its interaction with mTORC1/Ragulator/ Rag and Rheb small GTPases [32–35]. An alternative direct role of eukaryotic ATPases in membrane fusion has been previously proposed [36–39] and the V_O has been implicated in this process during exocytosis and insulin secretion in mammalian pancreatic β-cells [2, 8]. Moreover, V-ATPase with a3 and d2 isoforms is assembled in the osteoclast plasma membrane, and a direct role of the d2 isoform in the fusion of osteoclast progenitors has been described [2, 40–43]. Here, we discuss the current understanding of the structure of eukaryotic V-ATPase, focusing on the recently determined crystal structure of *S. cerevisiae* DF-subunit complex [44]. The finding of their interaction interface could reveal functional insights into coupling and regulation of all eukaryotic V-ATPases. In addition, we describe the emerging novel roles of V-ATPases in acidification of compartments, modulation of the function of critical cellular receptors as well as pH and nutrient sensing and signaling via its super-complexes.

In recent years, the V-ATPase has been implicated in the pathophysiology of a variety of human diseases including primary distal renal tubular acidosis accompanied by sensorial deafness [45], autosomal recessive osteopetrosis [41, 46], and autosomal recessive cutis laxa [47–49]. In addition, a role of the V-ATPase in cancer has recently emerged, since its increased expression at the plasma membrane correlates with the invasive characteristics of various malignant cells [50–53]. Based on these findings, perspectives and strategies in drug targeting to V-ATPase in human disease is discussed.

2 Structure of the Multi-subunit Eukaryotic V-ATPase

2.1 The Two Sector Composition of V-ATPase

Eukaryotic V-ATPases are multi-protein complexes composed by 14 different subunits $A_3B_3CDE_3FG_3Hac_xc'_Yc''_zde$. V-ATPase holoenzyme have a bipartite structure formed by a cytoplasmic V_1 ($A_3B_3CDE_3FG_3H$) and a membrane-embedded V_O ($ac_xc'_Yc''_zde$) sectors (Figs. 16.1 and 16.2). The stoichiometry of three V_O subunits ($c_Xc'_Yc''_z$) is unknown, although they are multiple. Both sectors are linked by connecting regions that are important for coupling between ATP hydrolysis in V_1 and proton translocation in V_O. These connecting regions consist of a central (D,F,d) and three peripheral (E,G) stalks, which are also important for reversible disassembly/ assembly of V-ATPase (Fig. 16.1a). Within two V_1V_O sectors, however, there are functionally identifiable "stator" ($A_3B_3EGCHae$) and "rotor" ($DFdc_Xc'_Yc''_z$) subcomplexes responsible for implementation of rotary mechanism of V-ATPase nanomotor [1–5, 12].
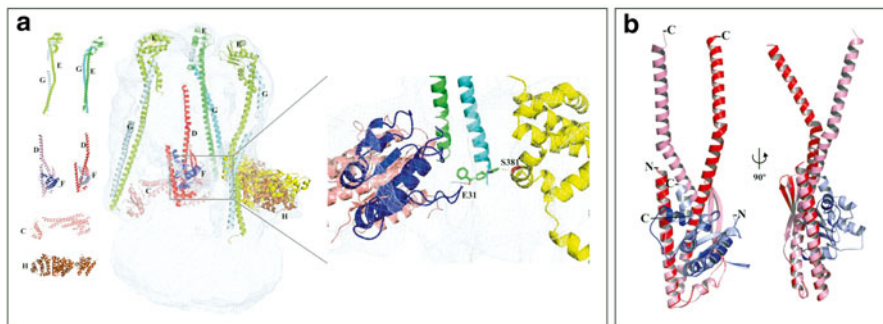
Fig. 16.1 Arrangement of the existing individual atomic subunit structures in the cryo-EM-map of the *S. cerevisiae* V-ATPase. (**a**) Subunits C (1U7L; *salmon*), H (1HO8, *brown*), and the D (4RND, *red*) and F assembly (4RND; *blue*) from *S. cerevisiae* were fitted into the cryo-EM map. The two conformations of EG subunits, the straight (4DL0; *green* and *cyan*) and more bent (4EFA; *lemon* and *pale cyan*) are fitted to the three peripheral stalks. The crystallographic structure of two conformations of subunit D in ensemble with the stalk subunit F of *S. cerevisiae* (*Sc*DF1 and *Sc*DF2) V-ATPase are shown. (*Insert*) Region of the EM-map showing the interaction of modeled subunit H (S381) (*yellow*) through the sulfhydryl cross-linker 4-(*N*-maleimido)benzophenone (MBP) (stick; *green*) to the *S. cerevisiae* subunit F1-94 (E31) [44]. Cartoon representation of the structures of the individual *S. cerevisiae* subunits C (1U7L; *salmon*), F1-94 (4IX9, *blue*), H (1HO8, *brown*) and EG in two conformations, straight (4DL0; *green* and *cyan*) and bent (4EFA; *lemon* and *pale cyan*). (**b**) Intermolecular interactions of the subunit DF-assembly. Superimposition of the *Sc*DF1 (*pink* and *blue*) with *Sc*DF2 molecule (*orange* and *grey*)

2.2   Catalysis and Energy Coupling in V_1 Sector

V-ATPases exist in a dynamic equilibrium between fully assembled holoenzyme and reversibly disassembled V_1 and V_O [13–19]. Depending on the energy state of the cell, this equilibrium can be rapidly shifted [16, 54]. Recently, *S. cerevisiae* and *M. sexta* V-ATPase holoenzymes have been isolated and its three-dimensional (3D) structure was shown using high resolution cryo-electron microscopy (cryo-EM) analysis [55–57]. The structure reveals that three A and B subunits form a hexagon with the nonhomologous regions at the top of subunit A [7, 55, 56, 58, 59]. The two nucleotide-binding subunits A and B alternate around a central cavity, which narrows toward its center and opens at both ends. Subunit D inside the cavity forms different interaction with domains of the three subunits A and B [12, 55, 56, 58]. The crystallographic structure of two conformations of subunit DF complex (*Sc*DF1 and *Sc*DF2) forming the central stalk of *S. cerevisiae* V-ATPase has been solved recently [44]. Subunit D in the complex consists of a long pair of α-helices, connected by a short helix ($_{79}$IGYQVQE$_{85}$) and a β-hairpin region, which is flanked by two flexible loops (Fig. 16.1b). The long pair of helices is composed of the N- and the C-terminal helix, respectively, and show structural alterations in the *Sc*DF1 and *Sc*DF2 structures. The subunit F consists of an N-terminal domain of four β-strands (β1-β4) connected by four α-helices (α1-α4). α1 and β2 are linked via the loop $_{26}$GQITPETQEK$_{35}$, which is unique in eukaryotic V-ATPases (Fig. 16.1a). Adjacent
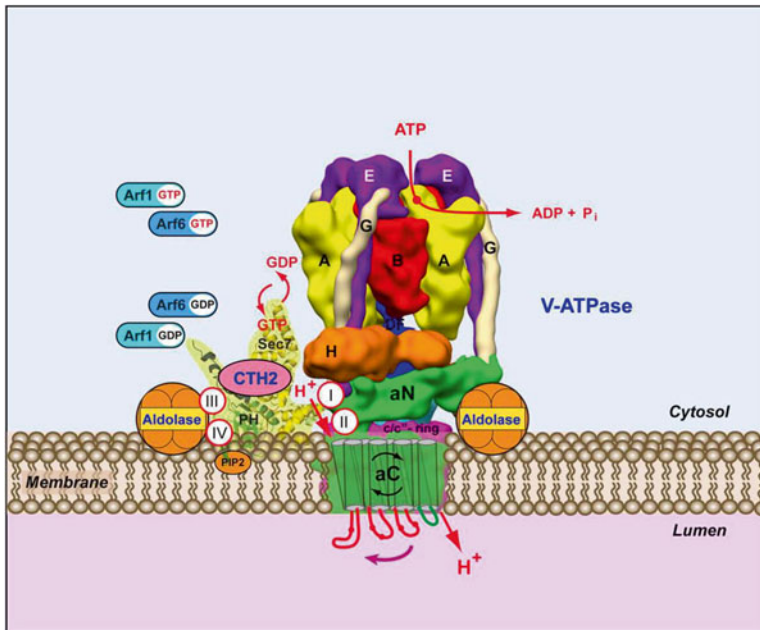
Fig. 16.2 Structure and composition of a novel V-ATPase/cytohesin-2/Arf1,6/aldolase signaling super-complex. The diagram shows the structure of the novel V-ATPase/cytohesin-2/aldolase/Arf1,6 super-complex localized on early endosomal (EE) membrane (see Fig. 16.3, *Complex 1*). It illustrates the binding site of cytohesin-2 (CTH2, in *yellow*) with an N-terminal tail of a2-subunit (a2N, in *green*) of V-ATPases [25, 29]. On the *left* is shown the CTH2 molecule interacting with a2N thorough Sec7-domain and with aldolase through PH-domain. *Roman numbers* indicate interfaces and affinities of interaction: (*I*) CTH2 with *a*2N(1–402) and (*II*) Sec7 domain with *a*2N(1–17) and *III, IV* CTH2 with aldolase [28, 167]. On the *right* is shown the aldolase molecule interacting with a2N of V-ATPase

to the N-terminal domain is a flexible loop (P95-D106), followed by a C-terminal α5-helix. A perpendicular and extended conformation of the α5-helix was observed in the two crystal structures and in solution X-ray scattering experiments, respectively (Fig. 16.1a, left) [44]. The concerted interaction of the DF complex, including the P95-D106-loop with helix α5 of subunit F and bended C-terminal helix of subunit D, may activate ATP hydrolysis in the catalytic A_3B_3 hexamer [44, 60].

The $_{26}$GQITPETQEK$_{35}$-loop of the subunit F is facing to the C-terminal serine (S_{381}) of subunit H, revealed to be involved in cross-linking subunit F of the V_1 (Fig. 16.1a, right) [60, 61]. The unique stalk of subunit H is characterized by a large, primarily α-helical N-terminal domain, which is forming a shallow groove connected by a four-residue loop to the C-terminal domain [62]. This arrangement led to the proposal, that in the process of V_1 and V_O dissociation the flexible C-terminal domain of subunit H moves slightly closer to the exposed $_{26}$GQITPETQEK$_{35}$-loop of subunit F, where it causes conformational changes, leading to an inhibitory effect of ATPase activity in the V_1 [44, 60]. ^{15}N-[^{1}H]

heteronuclear NOE studies on the subunit F revealed a rigid core formed by β-strands, β1–β4, and α2–α4. In comparison, the N- and C-terminal helices α1 and α5 with their adjacent loops $_{26}$GQITPETQEK$_{35}$ and $_{94}$IPSKDHPYD$_{102}$, respectively, are more flexible in solution [60]. The N-terminal helix α1 of subunit F and the bottom segment of subunit D are in proximity to subunit d, forming the tip of the central stalk and being in direct neighborhood of the proton translocating ring of c-, c′-, c″-subunits of V_O [60]. This area might be modified during the process of reversible assembly/disassembly of the V_1 and V_O, as shown previously. The higher flexibility of α1 in subunit F would allow to transmit the conformational change of subunit d during dissociation from the DF-heterodimer and also the movement of subunit H closer to F, through the neighboring $_{26}$GQITPETQEK$_{35}$-loop [44, 60].

In addition to the central stalk, the catalytic A_3B_3 hexamer is connected to V_O by three peripheral stalks with a different degree of twisting in the C-terminal and/or middle part (Fig. 16.1a, left) [1]. The subunit E and G, forming the peripheral stalk, are arranged in a ~150 Å long complex. The peripheral stalk is connected to the A_3B_3 through the globular C-terminus of subunit E, formed by α-helices and β-sheets, arranged as β1:α1:β2:β3:β4:α2 [63, 64]. This C-terminus is connected by a flexible loop region with N-termini of both subunit E and G, which are folded in a noncanonical, right-handed coiled coil. The coiled coil is disrupted by a bulge of partially unfolded secondary structure in subunit G, which provides the necessary flexibility of the peripheral stalk during detachment and assembly of the V_1 from the V_O (Fig. 16.1a, left) [63].

The reversible disassembly of the V_1 and V_O is initiated by the dissociation of subunit C [65]. As shown for its hydrated [66] and crystallized form [67] subunit C is a boot-shaped protein with an upper head domain formed from α-helices and β-strands (residues 167–262) and a globular foot domain (residues 1–55 and 320–392). Both domains are connected by an elongated helical neck domain (Fig. 16.1a, left) [67]. Location and orientation of subunit C in the enzyme enables its binding to actin [67], ADP/ATP nucleotides [68], and WNT-kinase [69]. These interactions are taking place through the foot domain allocated in proximity to the N-termini of an EG-heterodimer, as well as in neighborhood to the N-terminal region of subunit a (Fig. 16.1a, left). It was suggested that binding of ATP/ADP [68] or WNT-kinase dependent phosphorylation [69] of subunit C could alter the stability of an subunit EGC assembly by affecting its binding properties with either EG-heterodimer or with actin.

2.3   Structure of the V_O Enabling Proton Conduction

The proton conducting V_O sector of V-ATPase may be formed by five or six subunits and two accessory proteins. Indeed, *S. cerevisiae* V_O consists of six different subunits (a, c, c′, c″, d, and e). However, the c′ subunit gene was not found in mammalian genome, and thus, the mouse and human V_O are formed only by five subunits: a (a1, a2, a3, or a4), c, c″, d (d1 or d2), and e (e1 or e2). In turn, the mammalian V_O

also contains the two additional accessory subunits Ac45 and M8-9 [1, 5, 70]. Structurally, the V_O is composed of a ring of c- and c''-subunits (c/c'' ring) and the adjacent single copies of the a, e, Ac45, and M8-9 subunits. As suggested by the 3D map of the V_O from bovine clathrin-coated vesicles V-ATPase, the accessory subunit Ac45 interacts with the c/c'' ring from the luminal side [71].

On the other hand, the N-terminal cytosolic tail of subunit a (aN) is oriented parallel to the cytoplasmic surface of the membrane in the close proximity to the N-terminus of subunit H (Fig. 16.2) [1, 55]. Recent small-angle X-ray scattering studies of the N-terminal tail $a_{104-363}$, suggested the connection between the cytoplasmic N-terminal (aN) and the transmembrane C-terminal (aC) domains of subunit a (Fig. 16.2) [72]. This arrangement makes the aN of V-ATPase accessible for cytohesin-2 (CTH2) and Arf1, Arf6 small GTPases (Fig. 16.2), which is essential for signaling and trafficking of various receptors, including EGFR/ErbB receptors (Fig. 16.3) [1, 31, 72]. Although the structure and orientation of the aN is available, the transmembrane topology of its aC remains controversial (Fig. 16.2) [1, 26]. In yeast a six [73, 74], eight [75, 76], and nine [77, 78] transmembrane helix models have been proposed. According to the model with eight helices, both N- and the C-termini of the a subunit (Vph1p) are located in the cytosol, which is supported by the results showing interaction of phosphofructokinase-1 with the C-terminal tail of the human a4- and a1-isoforms [79]. The moving membrane part of the yeast V-ATPase "rotor" is a ring composed by the c-, c'-, and c''-subunits. The c- and c'-subunits are 16 kDa proteins, proposed to contain four transmembrane helices with two loops exposed to the cytosol, while the c''-subunit is a 23 kDa polypeptide with five putative transmembrane helices, two loops and a C-terminal tail exposed to the cytosol [80, 81]. Recently, cryo-EM observation of rotational states in *S. cerevisiae* V-ATPase has revealed the involvement of ten c-, c'-, and c''-subunits in the ring formation [56]. Each of these subunits is contributing two transmembrane helices to the inner ring and two helices to the outer ring. In addition, this study supported the eight transmembrane helices model of a-subunit [75, 76]. Remarkably, it was found that two of the helices are highly tilted and span the membrane where the a-subunit is in contact with the ring of ten c-, c'-, and c''-subunits, providing the new insights on the proton conducting mechanist of V_O sector [56]. The subunit composition and number of transmembrane helices in the c/c''-rings of mammalian V-ATPases are open questions. Recently, the first evidence for the position of subunit e within V_O has been provided [82].

The cryo-EM map of yeast V-ATPase revealed that a proton conducting channel is formed by the interface between transmembrane helixes of a-subunit and the ring of c-, c'-, c''-subunits. This channel is very narrow and occurs near the middle of the membrane region [12, 83]. It is important to note that the potent V-ATPase inhibitors *bafilomycin A1* (*BafA1*) and *concanamycin A* (*ConA*) bind to the interface between a-subunit and the ring of c-, c'-, c''-subunits inhibiting both rotary and proton-pumping mechanisms of yeast V-ATPase [84–86]. Although these compounds are very useful to analyze the role of V-ATPases in inside-acidic organelles of cultured mammalian cells [87, 88], they are unable to distinguish between V-ATPases with different isoforms, which are targeted to specific compartments.
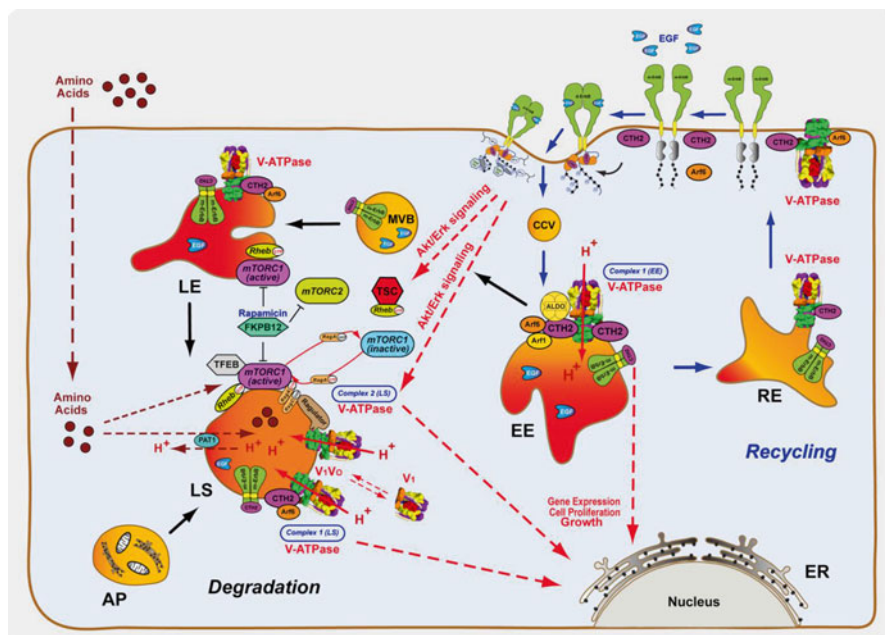
Fig. 16.3 Signaling and trafficking of EGFR/ErbB-receptors in endosomal/lysosomal pathway. Scheme shows signaling of epidermal growth factor (EGF) and trafficking of EGFR/ErbB-receptors in clathrin-dependent endocytosis and endosomal/lysosomal protein degradative pathway. The compartments are shown in yellow/red as follows: *CCV* clathrin-coated vesicles, *EE* early endosomes, *MVB* multi-vesicular bodies, *LE* late endosomes, *RE* recycling endosomes, *LS* lysosomes, *AP* autophagosomes, *ER* endoplasmic reticulum. Vesicular trafficking steps for the degradation branch are indicated with *black arrows* and for recycling branch with *blue arrows*. (*EE and super-complex-1*) Structure and composition of V-ATPase/CTH2/aldolase/Arf1,6 super-complex located in early endosomes. V-ATPase in this super-complex functions as pH-sensing and cytohesin-2/Arf1,6 signaling receptor which may regulate the trafficking and signaling EGFR/ErbB-receptors. (*LE/LS and super-complex-2*) Structure and composition of a novel V-ATPase/Ragulator/RagA/C/mTORC1/Rheb super-complex located in late endosomes and lysosomes. This complex is involved in sensing levels of amino acids and modulation of mTORC1-dependent downstream cellular programs and cell growth. Moreover, reversible association/dissociation of V_1V_O sectors of lysosomal V-ATPase is regulated by signaling of EGFR/ErbB-receptors through Akt/Erk pathway (*dashed red arrows*)

Therefore, there is an urgent need for the development of a new generation of inhibitors capable of selective inhibition of the subset of V-ATPases located at the specific compartments, such as Golgi, endosomes and lysosomes among others. Thus, an accumulating knowledge of high resolution structures of individual V-ATPase subunits as well as their interaction interfaces will be important for future developments of new generation of: (1) isoform and (2) organelle and/or (3) cell specific V-ATPase inhibitors (Figs. 16.1 and 16.2).

3 The Role of V-ATPases and Their Super-complexes in Acidification, Signaling, and Sensing

V-ATPase generates an electrochemical proton gradient, forming acidic intracellular compartments [1–5]. This enzyme is also targeted to the plasma membrane and is involved in extracellular acidification of specialized cells in kidney [89–91], epididymis [90, 92, 93], and bone [40, 74, 94–97]. It also acidifies extracellular environment in metastatic cancer cells [50–53]. The regulation of V-ATPase is achieved by the following three major processes: (1) modulation of acidification by the chemiosmotic mechanism; (2) targeting to specific organelles; and (3) regulation of the enzyme activity through reversible assembly/disassembly V_1 and V_O.

3.1 Chemiosmotic Process

The electrochemical proton gradient generated by V-ATPase consists of a proton gradient (ΔpH) and membrane potential ($\Delta\Psi$). Using a FRET approach, the values $\Delta pH = 2.2$ units and $\Delta\Psi = 27$ mV were experimentally determined in intracellular organelles [98]. Acidification is predominant function of V-ATPase, which depends on its coupled function with Cl^-/H^+- and Na^+/H^+-exchangers as an important chemiosmotic mechanism regulating acidification of intracellular organelles [99–102].

3.2 Process of Subunit-Specific Targeting of V-ATPase

The *S. cerevisiae* V-ATPase is targeted by the two *a*-subunit isoforms Vph1p and Stv1p to the vacuole and Golgi/endosomes, respectively [103, 104]. Studies with chimeric proteins revealed that the targeting information is located in the cytosolic N-terminal domain of the a-subunit [104]. Similarly, in mammalian cells, localization of V-ATPase in endocytic and exocytic compartments and targeting to the plasma membrane depend on *a*-subunit isoforms (Figs. 16.2 and 16.3) [1–4, 94]. Of four subunit *a* isoforms (*a*1, *a*2, *a*3 and *a*4) [1–4], V-ATPase with a1 isoform is specifically targeted to presynaptic membranes and exocytic synaptic vesicles in mammalian neurons [105, 106]. Studies with neurosecretory PC12 cells revealed that both *a*1 and *a*2 regulate the acidification and neurotransmitter uptake and release by exocytic vesicles [107]. In contrast, V-ATPase with *a*1 is targeted to the endocytic pathway in brain microglial cells, and plays role in the fusion between phagosomes and lysosomes during phagocytosis, a process of microglial-mediated neuronal degeneration [108].

The *a*2 isoform targets V-ATPases to early endosomes of the endocytic pathway of kidney proximal tubule epithelial cells [25, 26, 30, 109, 110]. In these cells, the overexpressed recombinant *a*2-isoform (*a*2-EGFP) is also targeted V-ATPase to

endosomal compartments [31]. However, in cultured osteoclast cells and B16 cells, both endogenous *a*2 and a1 are targeted to secretory vesicles of Golgi complex in the exocytic pathway [94, 107]. Similarly, over-expression of recombinant *a*2 isoform (*a*2-EmGFP) in neuroendocrine PC12 cells targets V-ATPase to the Golgi apparatus [94, 107].

The V-ATPase with *a*3 is a lysosome specific enzyme of osteoclast progenitor. During differentiation, the V-ATPase with *a*3 is targeted and localized to the plasma membrane of osteoclasts. The osteoclast V-ATPase is involved in bone reabsorption and its defect caused infantile malignant osteopetrosis in humans [40, 74, 94–97]. V-ATPase with *a*3 is also targeted to the plasma membrane of breast cancer cells and plays an important role in metastatic processes [50–53]. Phagosomes in macrophage also acquire the same enzyme from lysosomes [111], whereas it is specifically targeted to insulin secretory granules of pancreatic β-cells [2, 94].

The V-ATPase with *a*4 is highly abundant in epididymis and kidney, where they are specifically targeted to the apical plasma membrane of epididymal clear cells and kidney collecting duct intercalated cells [89–93], indicting that this V-ATPase is involved in sperm-maturation [92, 93] and maintenance of acid-balance [90, 91], respectively. These results indicate that the *a*-subunit isoforms differentially target V-ATPase to the plasma membrane or intracellular compartments of the endocytic/exocytic pathways. In turn, the specific targeting and assembly of the enzyme may modulate acidification of extracellular milieu and intracellular organelles [1–4, 93, 112].

3.3   Reversible Assembly/Disassembly of V-ATPase

The regulation of V-ATPases by assembly/disassembly of the V_1V_O sectors was first described in response to ceased feeding in *M. sexta* [17, 18] and in response to glucose depletion in *S. cerevisiae* [13, 14, 19]. In *S. cerevisiae*, assembly/disassembly of the V_1V_O may be regulated by both *a*-isoform (Vph1 or Stv1) and E subunit. Evidence for the role of the E subunit in assembly was first obtained from yeast/mouse hybrid V-ATPase [113, 114]. A null mutant of yeast subunit E expressing the mouse (E1 or E2) is functional, indicating that this hybrid V-ATPase with E1 or E2 is functional as proton pump. However, assembly of the hybrid with E1 became less dependent on glucose [114]. Furthermore, a domain between residues K33 and K83 of *S. cerevisiae* subunit E could be replaced by the corresponding region of mouse E1 [114, 115]. Alanine scanning mutations revealed that the residue E44 of yeast subunit E is a key amino acid in regulation of subunit assembly and thus enzyme activity [115].

In *S. cerevisiae*, the reversible assembly and disassembly of V_1V_O sectors is controlled by two distinct mechanisms. While the assembly requires the cytosolic RAVE/Rab-Connectins complex (Rav-1, Rav-2, and Skp1), the disassembly process involves the cytosolic microtubular network [1, 2, 4, 5, 15, 116]. Recent study revealed, that RAVE/Rab-Connectins in yeast is an *a*-isoform specific complex, which is necessary for assembly of V-ATPase with Vph1p but not with Stv1p [117].

Moreover, the reversible assembly/disassembly of the yeast V_1 and V_O is also controlled by the direct interaction of V-ATPase with cytosolic aldolase, a central enzyme of the glycolysis (Fig. 16.2). The assembly/disassembly and interaction with aldolase is modulated by the Ras/cAMP/PKA pathway [118], suggesting that V-ATPases may act as a cytosolic glucose-sensor (Fig. 16.2) [119–121]. However, in mammalian kidney proximal tubule cells, glucose regulation on V-ATPase is modulated by the phosphatidylinositol 3-kinase (PI3K) pathway [21]. In dendritic cells, the assembly of V_1V_O is also regulated through the PI3K/mTOR-dependent pathway and is critical for lysosomal acidification, protein degradation, and antigen presentation (Fig. 16.3) [20, 24]. Thus, it is generally accepted that various regulatory pathways are involved in controlling assembly/disassembly of V-ATPase in eukaryotic cells and, therefore, in modulation of its function as well as acidification of intracellular organelles and extracellular milieu [22].

3.4 Novel Role of V-ATPases in Regulation of Signaling and Trafficking of Cellular Receptors

A novel role of V-ATPases in regulation of signaling, trafficking, and degradation of various cellular receptors has emerged recently. Endocytosis is an essential cellular process that is used by eukaryotic cells for the internalization of various receptors localized in the plasma membrane. As shown previously, the clathrin-dependent endocytosis pathway mediates internalization of Fz/LRP6, PRR, Notch, transferrin, megalin/cubilin, and EGFR/ErbB receptors among others (Fig. 16.3) [1, 31, 122]. Mounting evidence indicates that the V-ATPase is not only establishing the acidic pH in endocytic organelles but is also functioning as a cytohesin-2/Arf1,6 small GTPases signaling receptor (Fig. 16.2). Moreover, the V-ATPase is involved in direct interactions with critical cellular receptors, and thus, could modulate their signaling, traffic, and degradation along the endocytic pathway (Fig. 16.3). These emerging roles of V-ATPase will be discussed below.

3.5 V-ATPase and Epidermal Growth Factor Receptors (EGFR/ErbB's)

The epidermal growth factor receptor (EGFR) was among the first discovered growth receptors that regulate crucial cell biological processes including cell proliferation [123, 124]. The EGFR/ErbB-receptors (EGFR/ErbB's) family comprises four members (EGFR/ErbB1, ErbB-2, ErbB-3, and ErbB-4) and are involved in the development of a variety of cancers [124–127]. Activation of EGFR/ErbB-receptors by extracellular EGF ligand promotes their hetero-dimerization with subsequent activation of TK-domains and tyrosine trans-phosphorylation of the cytoplasmic tail.

However, the cytoplasmic proteins that could modulate EGF-induced activation and signaling of EGFR/ErbB-receptors were largely unknown.

Cytohesin-2 (CTH2) has been identified recently as a cytoplasmic activator of EGFR/ErbB-receptors (Fig. 16.3) [128, 129]. CTH2 enhances trans-dimerization and activation of EGFR/ErbB's by direct binding with TK-domains of dimerized receptors and by facilitating conformational changes and trans-phosphorylation of these domains. Figure 16.3 illustrates the signaling of the epidermal growth factor (EGF) through EGFR/ErbB's localized in the plasma membrane and early endosomes. The crucial role of CTH2 in hetero-dimerization of these receptors is also indicated. The figure also shows the V-ATPase dependent trafficking and signaling of EGFR/ErbB-receptors through the clathrin-dependent endocytosis endosomal/lysosomal protein degradation pathway. In particular, a novel important role of the assembly/disassembly of V_1V_O and V-ATPase-dependent late endosomal/lysosomal acidification in EGFR/ErbB-receptors function has been recently revealed [23]. It was demonstrated that EGF/EGFR-dependent signaling promotes the rapid recruiting of cytosolic V_1 sectors of the V-ATPase and increases its assembly with V_O on late endosomal/lysosomal compartments. This assembly in turn increases V-ATPase driven lysosomal acidification, protein degradation, and release of amino acids needed for Rheb(GTP) and mTORC1 activation (Fig. 16.3). V-ATPase is playing an indirect role in EGF-dependent activation of mTORC1 signaling pathway by modulating the assembly/disassembly of V_1 and V_O [32]. This is the first evidence showing the functional assembly of V-ATPase in response to the signaling of EGFR/ErbB-receptors. In this way, V-ATPase regulates mTORC1 signaling and trafficking EGFR/ErbB-receptors within the endosomal/lysosomal protein degradation pathway (Fig. 16.3) [23, 32]. Thus, V-ATPase-dependent acidification and cytohesin-2/Arf1,6 small GTPases signaling (Fig. 16.2) may play a key role in the modulation of EGFR/ErbB-receptors function, and is pivotal for the sustained signaling, recycling, or degradation (Fig. 16.3).

3.6   V-ATPase, Insulin-Like Growth Factor-1 Receptor (IGF-1R), and Heme-Responsive Gene 1 (HRG-1) Protein

Both the growth hormone (GH) and insulin-like growth factor 1 (IGF-1) exert powerful control over lipid, protein and glucose metabolism. The function of GH/IGF-I signaling pathway is associated with longevity, and thus, aging related morbidities including diabetes and cancer [130, 131]. This pathway also plays roles in maintenance and repair of muscles [132]. Signaling by insulin-like growth factor receptor (IGF-1R) controls expression of heme-responsive gene 1 (HRG-1) that encodes a 16 kDa transmembrane protein. A recent study revealed specific targeting of this protein to early endosomes and its direct interaction with V-ATPase c-subunit [133]. Increased expression of HGR-1 enhances V-ATPase activity in the plasma membrane, lowers the extracellular pH and activates pH-dependent matrix metalloproteinases. HRG-1 also increases endosomal V-ATPase activity, which promotes

trafficking of the IGF-1R, β1-integrin and glucose transporter-1 (GLUT-1) with concomitant increase of glucose uptake, cancer cell growth, migration and invasion. Thus, HRG-1 may represent a novel target for selectively disrupting V-ATPase activity and the metastatic potential of cancer cells [133, 134].

3.7   V-ATPase and Frizzled (Fz) and Low-Density Receptor-Related Protein (LRP6) Receptors

The Wnt/β-catenin, Wnt/PCP(planar cell polarity) and Wnt/Ca^{2+} signaling pathways are essential mechanisms that control embryonic tissue development, homeostasis, cell proliferation, polarity, and apoptosis [135, 136]. They are strongly linked to the development of a variety of human diseases including metastatic cancers [135, 136]. The direct role of V-ATPase in regulation of Wnt/β-catenin and Wnt/PCP signaling pathways has been uncovered recently [137, 138]. It was shown that signal transmission after association of Wnt ligands with Fz/LRP6 co-receptors requires direct interaction of LRP6 with an accessory M8-9 subunit of V-ATPase, also called V-ATPase lysosomal accessory protein-2 (ATP6AP2). This interaction takes place in early endosomes and the ATP6AP2 subunit acts as an adaptor that brings together V-ATPase and the Wnt/Fz/LRP6 receptor complex. Thus, both direct and electrochemical regulation by V-ATPases are involved in signaling of Wnt/Fz/LRP6 in endosomes involved in the protein degradation pathway.

3.8   V-ATPase and (Pro)renin Receptor (PRR)

The (pro)renin receptor (PRR), a single transmembrane cell surface receptor, plays a central role in activating the local renin-angiotensin system. Binding of prorenin to PRR induces its conformational change, allowing conversion of angiotensinogen to angiotensin-I, which is subsequently converted to angiotensin-II by an angiotensin-converting enzyme [139, 140]. However, an angiotensin-independent function of PRR has also been recently reported, which was identified as an accessory ATP6AP2 subunit of V-ATPase [137, 138, 140, 141]. Tissue-specific conditional knockout experiments confirmed a role of PRR/ATP6AP2 in assembly of V-ATPase in murine cardiomyocytes [142]. Importantly, the level of prorenin is elevated during diabetes and over-activation of PRR is associated with development of hypertension and diabetic kidney disease [122, 143]. The role of PRR in kidney function and its association with diabetes and hypertension has been recently reviewed [122, 140, 141, 143]. Thus, future studies in this area could lead to the novel therapeutic approaches for the treatment of hypertension, diabetes, and their complications [122].

3.9 V-ATPase and Notch Receptor

The cell-to-cell signaling by the Notch receptor pathway is critical during development and tissue regeneration for controlling the balance between cell proliferation and apoptosis. Pathological loss of regulation of Notch receptor signaling is also a hallmark of different cancers [144, 145]. Activation of the Notch receptor by ligands gives rise to its cleavage by γ-secretase-mediated intra-membrane proteolysis followed by activation of specific target genes. Surprisingly, recent studies revealed that V-ATPase driven acidification may control two opposite processes in the Notch signaling in *Drosophila*: (1) lysosomal degradation and loss of regulation of Notch receptors; and (2) γ-secretase-mediated Notch receptor activation in early endosomes [146]. Moreover, Notch receptor signaling is also controlled by Rabconnectin-3A/B (Rbcn-3) through regulating V-ATPase both in *Drosophila* and mammalian cells [147, 148]. It is noteworthy that mammalian Rbcn-3 protein is a homolog of yeast Rav-1, which forms a part of the RAVE (Rav-1, Rav-2, and Skp1) complex. Interaction of Rav-1 with V-ATPase is essential for reversible assembly/disassembly of the yeast V_1 and V_O [15, 116, 149]. Similar to Wnt-signaling, these studies also revealed two mechanisms of Notch-signaling modulation by V-ATPase in mammalian cells: (1) through subsequent Rbcn-3/V-ATPase interaction and (2) V-ATPase-driven acidification leading to activation of γ-secretase [138, 146–148].

3.10 V-ATPase as pH-Sensor and Cytohesin-2/Arf Small GTPases Signaling Receptor

The Arf-family small GTPases belong to the Ras-superfamily that are involved in regulation of a great variety of cellular pathways [150]. These GTPases function as "molecular switches" and the transition between "on" and "off" is mediated by a GDP/GTP cycle. In particular, activation of Arf small GTPases is accomplished by the cytohesin-family of guanine nucleotide exchange factors (GEFs). Cytohesin-family GEFs include cytohesin-1, cytohesin-2 (CTH2) (also known as ARNO), cytohesin-3 (also known as GRP1), and cytohesin-4. The generally accepted functions of cytohesin/Arf small GTPases are regulation of organelle biogenesis, modulation of vesicular trafficking, and actin cytoskeleton remodeling [151–153]. However, cytohesins have also emerged recently as central modulators of signaling and trafficking of plasma membrane receptors including: (1) EGFR/ErbB [128, 129], (2) insulin-receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) [154–158], (3) VEGF-2R [159], and (4) integrins [160, 161]. In particular, cytohesin-1 and -2 have been identified as activators of EGFR/ErbB's that are involved in regulation of cell proliferation and oncogenesis [128, 129]. Cytohesins are also crucial downstream effectors for the IR and IGF-1R signaling cascade involved in regulation of calorie restriction pathway, longevity, and aging process [154–158, 162–164].

Previously, our laboratory focused on studies of the functional interplay between V-ATPase and cytohesin-2/Arf1,6 small GTPases in endosomal/lysosomal protein degradation pathway [30, 109, 165, 166]. The cytohesin-2 and Arf1, Arf6 are specifically targeted to early endosomes of this pathway and co-localized with V-ATPase in kidney proximal tubule cells (Figs. 16.2 and 16.3) [30, 109]. Moreover, subsequent work from our laboratory revealed that V-ATPase itself functions as a novel long-sought pH-sensor or pH-sensing receptor. In particular, V-ATPase containing a2 isoform is specifically targeted to early endosomes and directly interacts with cytohesin-2 (CTH2) in acidification-dependent manner, suggesting that the a2 is a putative pH-sensing receptor (Fig. 16.2) [25, 26]. According to this concept, V-ATPase is not only responsible for proton pumping and the generation of a pH gradient but also involved in sensing the levels of acidification and transmitting this information through membrane. Moreover, other isoforms (a1, a3, and a4) were also found to interact with CTH2 [167]. Taken together, these results suggest that pH-sensing by V-ATPases and interaction with cytohesin-2/Arf1,6 small GTPase is a general phenomenon, which may take place in other acidic organelles of both the exocytotic and the endocytic pathways. We have uncovered the molecular details of unexpected function of V-ATPases, as an evolutionarily conserved pH-sensing and cytohesin-2/Arf1,6 small GTPases signaling receptor (Fig. 16.2) [1, 2, 25–27, 31, 167].

4   Functions of V-ATPase in Normal and Disease States

In this section, we discuss roles of V-ATPases in regulating vesicular trafficking and the development of various disease states. First, our focus is the role of V-ATPases in cancer development and metastasis process. Second, we analyze its role in modulation of exocytic/secretory pathway during pathogenesis of diabetes and bone diseases. Third, we discuss the roles of V-ATPases in regulating two novel super-complexes localized in endocytic or endosomal/lysosomal protein degradation pathway (Fig. 16.3). We also discuss their roles in cancer as well as aging and age-related diseases.

4.1   V-ATPase in Cancer Development and Metastasis

Human and mouse a1 and a2 are expressed ubiquitously under physiological conditions, whereas a3 and a4 are specifically expressed and targeted to the plasma membranes of bone osteoclast and kidney intercalated cells, respectively [73, 92, 93, 168, 169]. Previous studies suggested that the a subunit targets V-ATPase to different compartments and plasma membranes as well as senses the pH-gradient formed during acidification (Fig. 16.2) [1, 2]. V-ATPases with specific a-isoforms have been detected at the plasma membrane of the breast, ovarian, and prostate cancer cells. The role of V-ATPase dependent extracellular acidification was studied extensively in development and invasive phenotype of these metastatic tumors.

4.1.1   Breast Cancer

Early studies have demonstrated that V-ATPases are expressed in the plasma membrane of invasive cancer cells [170]. V-ATPase was found to be more prominently expressed in the highly metastatic MDA-MB231 breast cancer cells, which are more invasive and migratory than the less metastatic MCF-7 breast cancer cells. Moreover, inhibition of V-ATPase by *BafA1* significantly reduces the in vitro invasion of MDA-MB231 but not MCF7 cells [50]. These results indicate that targeting of V-ATPase to the plasma membrane is correlated with metastatic phenotype of the breast cancer cells. However, the mechanism of V-ATPase function and the subunits involved in its targeting to the plasma membrane remain obscure. Further studies have focused on the role of the a-subunit isoforms in cancer. Although all four isoforms are found in MDA-MB231 but not MCF7 cell lines, the expression levels of a3 and a4 are significantly higher in metastatic MDA-MB231 than in non-metastatic MCF-7 cells. In addition, siRNA knockdown experiments further demonstrated the role of a3 and a4 isoforms in targeting V-ATPase to the plasma membrane and invasive phenotype of MDA-MB231 cells [51]. Similar role of a3 was found in invasiveness of MCF10CA1 but not MCF10a breast cancer cells [52]. Finally, the recent studies demonstrated that invasiveness of breast cancer cells could be modulated by cell-impermeable molecules targeting extracellular parts of V-ATPase: (1) a biotin-conjugated *BafA1* or (2) the monoclonal antibody directed against the V5 epitope constructed on the extracellular loop of V_O c-subunit [53].

Other roles of V-ATPase in breast cancer development and metastasis have been revealed recently. First, V-ATPase is required for trafficking of Rab27B small GTPase dependent pro-invasive secretory vesicles which promote an invasive growth and metastasis in estrogen receptor (ER) alpha-positive breast cancer patients. Therefore, a role of V-ATPase in invasive growth and metastasis of ER-alpha-positive breast cancer has been suggested [171]. This study demonstrated that inhibiting V-ATPase by *BafA1* or silencing of a1 or d subunits might be an effective strategy for blocking Rab27B-dependent pro-invasive secretory vesicles which are involved in secretion of pro-invasive growth regulators. Second, V-ATPase driven acidosis of tumors may also control the pro-apoptotic protein Bnip3 death pathway [172]. Accordingly, it was reported that pharmacologic inhibition of V-ATPase with *BafA1* could effectively activate Bnip3 pathway, promote death of breast cancer cells and significantly reduced tumor growth in MCF7 and MDA-MB-231 mouse xenografts in vivo. Importantly, the combined treatment of mice bearing the breast MDA-MB-231 xenografts with *BafA1* and ERK1/2 inhibitor *sorafenib* has potentiated action of two inhibitors for tumor regression. These results present a novel mechanism to kill cancer cells and reverse resistance of breast hypoxic tumors. Third, breast cancer invasive cells are resistant to anoikis, a specific type of apoptosis promoted by loss of cell–matrix contact. A recent study demonstrated, that triggering of anoikis by V-ATPase inhibitor *archazolid* is promising therapeutic approach to reduce metastasis of breast cancer cells in mouse model in vivo [173]. Forth, very recent study has identified a novel tumor-metastasis related gene 1 (TMSG1) as a regulator of activity of V-ATPase and secreted metalloproteinase-2 in breast cancer cells [174].

All together, these studies suggest that V-ATPase could modulate invasiveness and breast tumor metastasis due to acidification of extracellular milieu and subsequent activation of metalloproteases. On the other hand, V-ATPase also controls apoptotic cell death and regulates vesicular trafficking and secretion of pro-invasive growth factors. Importantly, in breast cancer cells both invasiveness and metastasis could be controlled by targeting extracellular part of plasma membrane V-ATPase [175–177].

4.1.2 Ovarian Cancer

The role of V-ATPase in invasiveness and metastasis has also been recently addressed in ovarian carcinoma. Particularly, the expression, targeting, and function of a-subunit isoforms were studied in ovarian cancer tissues as well as in ovarian cancer A2780, SKOV-3, TOV-112D cell lines [178]. The a2-isoform is predominantly expressed in these cells and targeted V-ATPase to the plasma membrane. Under physiological conditions, a2-isoform is predominantly located in early endosomal compartment or Golgi and, thus, it relocates to the plasma membrane during tumorigenesis and metastasis. This study also reveals co-association of a2-isoform with cortactin, a protein involved in invasion of tumor cells. Targeting of a2 with monoclonal antibody reduces the activity of matrix MMP-2 and MMP-9 metalloproteinases and invasiveness of these ovarian cancer cells. These findings suggest that a2-isoform could be promising target for developing novel therapeutic anti-V-ATPase antibodies against ovarian carcinoma [178]. Finally, an important role of the a2-isoform derived secretory peptide in inflammation, angiogenesis, and tumorigenesis was also proposed [179–182].

4.1.3 Prostate Cancer and Tumor Angiogenesis

Angiogenesis is recognized as one of the hallmarks of cancer which enable tumor growth and metastatic dissemination [127]. Moreover, tumors are currently recognized as abnormal organs consisted of a complex mixture of the cells interacting and signaling with each other and required stable supply of nutrients and oxygen for their needs [183]. The cancer-induced neovascularization is triggered by pro-angiogenic signaling and cell-to-cell cross talk. In particular, plasma membrane V-ATPase was implicated in regulation of intracellular pH and migration of microvascular endothelial cells [184, 185]. Moreover, V-ATPase is taking part in cross talk and regulation of communication between microvascular endothelial and metastatic cells promoting acidification of extracellular space and favors protease activity [186]. A different mechanism of cross talk in metastatic prostate cancer cells involves regulation of V-ATPase by pigment epithelium-derived factor (PEDF), a potent endogenous inhibitor of angiogenesis. Thus, PEDF was identified as novel regulator of V-ATPase and suggested the mechanism of its inhibition of prostate tumor growth.

In summary, during last decade V-ATPase emerged as a crucial enzyme for tumorigenesis and metastatic phenotype of breast, ovarian, and prostate cancers. However, during last years the pathophysiological role of V-ATPase in the development of other tumors including: melanoma [187], hepatocellular carcinoma [188], oral squamous carcinoma [189], esophageal squamous carcinoma [190], non-small-cell lung cancer [191], gastric cancer [192], colon cancer [193], and pancreatic cancer [194] were also studied.

4.2   V-ATPase in Insulin Secretion and Diabetes Mellitus

V-ATPases play also essential roles in specific organelles of differentiated cells, especially those involved in exocytosis. The secretory granules have acidic luminal pH (5.0–5.5), important for condensation and maturation of their content. It has been suggested that the inside acidic pH or proton gradient across organelle membranes is involved in fusion of the vesicles to target membranes. Interesting question is which a-subunit isoform is expressed in secretory vesicles of mammalian cells. We have focused on the role of a-isoforms in insulin secretion, since previous studies suggested that V-ATPases may be pertinent for the insulin secretion [8, 195]. Of four isoforms, the a3 was detected in almost all cells in pancreatic Langerhans islet, and localized to the membranes of insulin containing granules in β-cells. Consistent with this finding, oc/oc mutant mice, homozygous 1.6 kb deletion of the a3-locus, exhibited reduced insulin secretion into blood upon glucose administration. However, the mutant β-cell contained essentially the same amount of mature insulin as the wild-type cell. Thus, the secretion of insulin was impaired in mutant β-cells. These results suggest that the a3-isoform has a direct function in exocytosis, possibly for fusion of the secretory vesicles to plasma membranes. The human ATP6i gene encoding the a3-isoform was mapped to a chromosome 11q13, and is located about 200 kb apart from LRP5 locus, which shows strong linkage to the disease. It is highly likely that alteration of ATP6i could also contribute to Type 1 diabetes [196]. We have identified that V-ATPase with a3 is highly expressed in endocrine tissues such as adrenal, parathyroid, thyroid and pituitary gland. Thus, a3 may be commonly involved in exocytosis of endocrine tissue and play an important role in the pathogenesis of endocrine and metabolic diseases including diabetes mellitus [8].

4.3   V-ATPase in Bone Homeostasis and Diseases

Bone homeostasis is maintained through the equilibrium between bone genesis and resorption by osteoblast and osteoclast, respectively. Defects of these cells are related to bone diseases such as osteopetrosis and osteoporosis. Osteoclast generates proton flux into bone resorption lacuna to mobilize bone calcium. This

acidification is carried out by V-ATPases, which are localized in the osteoclast plasma membrane. We have shown that a3 and d2 subunits are components of the osteoclast enzyme [40, 75]. Mammalian subunit d has two isoforms: ubiquitous d1 isoform and osteoclast/kidney/epididymis specific d2 isoform [40]. In contrast to the plasma membrane localization in osteoclast, the a3 isoform was found in late endosome/lysosome in NIH3T3 or RAW264.7 cells.

Murine macrophage line RAW264.7 can differentiate to multinuclear osteoclast-like cells. Upon stimulation with RANKL (Receptor Accepter Nuclear κB Ligand) from osteoblast, RAW264.7 cell differentiate into multinuclear osteoclast. During the differentiation, V-ATPase containing a3 isoform was transported to the cell periphery together with lysosome marker proteins, and finally assembled to the plasma membrane. The d2 isoform was also induced and assembled to the plasma membrane V-ATPase. Thus, secretory lysosomes should be involved in the formation of osteoclast plasma membranes. The splenic macrophage (from a3 knockout mice) lacking the a3-isoform could differentiate to multinuclear cells, which express osteoclast marker enzymes and V-ATPase with d2 and a1 or a2 isoforms [40, 197]. However, the multinuclear cell could not adsorb calcium phosphate, indicating that V-ATPase with d2/a2- or d2/a1-subunit isoforms could not perform the function of that with d2/a3-subunit isoforms containing V-ATPase.

In summary, we have studied the targeting and function of a3-isoform both in endocrine tissues and in bone osteoclasts. Remarkably, while in pancreatic β-cells a3 is targeting the V-ATPase to the membranes of insulin containing secretory granules of exocytic pathway, in bone osteoclast the V-ATPase with a3-isoform is expressed in plasma membrane and late endosomes/lysosomes of endocytic pathway. However, our results strongly suggest that V-ATPase a3 is localized into specialized secretory lysosomes in osteoclasts. These specialized lysosomes are not functioning as common endocytic lysosomes, but instead are transported to cell periphery and fused with plasma membrane, using the mechanism similar to exocytosis. In accordance with the pivotal role of V-ATPase in bone homeostasis, multiple mutations of the a3-isoform give rise to diseases of bone resorption and are associated with osteopetrosis in both mice models and humans [41, 46, 95, 198, 199].

4.4 V-ATPase and mTORC1/Ragulator/Rag/Rheb Small GTPases Super-complex in Cancer, Diabetes, and Age-Related Diseases

The mammalian target of rapamycin (mTOR) is a large cytosolic serine-threonine kinase that controls cellular growth and metabolism. Under physiological conditions both mTORC1 and mTORC2 are involved in neonatal autophagy and survival as well as development of obesity and aging processes in adulthood. Abnormal function of mTORC1 and mTORC2 are implicated in the pathogenesis of many diseases including cancer, diabetes, age-related diseases, aging, and

longevity [34, 35, 163, 200–204]. The mTOR belongs to the superfamily of phosphatidylinositol-3 kinase related-kinases (PI3KK) that forms the core of two functionally distinct complexes: mTORC1 and mTORC2. As mTORC1 primarily responds to the levels of amino acids, oxygen, energy and cellular stress stimuli, mTORC2 plays a central role in the growth factor and insulin signaling cascades (Fig. 16.3) [33–35]. mTORC2 also regulates cytoskeleton function, metabolism, and cell survival [35, 205]. V-ATPase has been identified recently as an important component of the mTORC1 regulatory super-complex and signaling pathway [32]. This super-complex, formed by V-ATPase, Ragulator, Rag, mTORC1, and Rheb, is associated with late endosomes and lysosomes of the protein degradation endocytic pathway (Fig. 16.3) [32, 206, 207]. Localization of mTORC1 on the late endosoaml/lysosomes membrane is also critical for its activation as a multi-functional serine–threonine kinase and is regulated by two types of small GTPases: (1) Rheb GTPase (Ras homolog enriched in brain); and (2) Rag GTPases. It is well recognized, that Rheb is a potent activator of mTORC1, which transmit signals of growth factors, oxygen, energy supply, and stress via the tuberous sclerosis complex (TSC), acting as a GTPase activating protein (GAP) for Rheb small GTPase.

Although amino acids are known to modulate cell growth and homeostasis, the molecular aspects of their regulation of mTORC1 function remained elusive. However, Sabatini and coworkers have shown recently that V-ATPase is a major player in the amino acids dependent recruitment, activation and signaling of mTORC1 [32]. V-ATPase is involved in sensing the levels of intra-lysosomal amino acids through the direct interaction with the Ragulator complex, that acts as a GTPase GTP/GDP-exchange factor for Rag small GTPases [33, 208]. They have proposed that the primary function of amino acid-dependent V-ATPase/Ragulator/Rag-signaling complex is to promote recruiting mTORC1 to lysosomal membrane, and thus trigger the TSC/Rheb-driven "ignition key" for the activation of the kinase activity of lysosomal mTORC1 complex. In their scenario, V-ATPase plays a direct role in intra-lysosomal sensing of amino acids and trans-membrane signaling to mTORC1.

Recent studies demonstrated, that mTORC1 is also activated by EGF via EGFR-receptor signaling pathway (Fig. 16.3). Then, the activation of mTORC1 involves in the Akt/Erk activation, TSC complex inhibition, and Rheb(GTP) formation. In contrast to amino acid-induction studies [32], EGF signaling pathway does not accompany mTORC1 recruitment from the cytosol and its translocation to the lysosomal membrane [23, 32]. Instead, the EGF signaling promoted the rapid recruitment of V_1 sectors and increased assembly of ATPase in late endosomal/lysosomal compartments. Thus, the novel role of V-ATPase in regulation of mTORC1 signaling and trafficking EGFR/ErbB-receptors within the endosomal/lysosomal protein degradation pathway is a crucial mechanism controlled by the assembly/disassembly of the V_1 and V_O (Fig. 16.3) [23, 32].

In summary, lysosomes and related organelles play a regulatory role in cellular protein degradation and energy production using V-ATPase/mTORC1 "sensing machinery" to monitor both lysosomal and cytosolic amino acid content as indicator of nutritional status of the cell. This physiological information is further

communicated to the nucleus to activate the gene expression programs allowing lysosomes to regulate their own function [209]. This quality control process is declining over life span, contributing to cancer and aging associated diseases, and thus, V-ATPase/mTORC1/Ragulator/Rag/Rheb small GTPases super-complex is considered as an important drug target.

4.5 V-ATPase and Cytohesin-2/aldolase/Arf1,6 Small GTPases Super-complex in Cancer, Diabetes, and Age-Related Diseases

Our recent studies uncovered, that in addition to its primary role as a proton-pump, V-ATPase also functions as a pH-sensing and cytohesin-2/Arf1,6 small GTPases signaling receptor (Fig. 16.2) [1, 2, 25, 27, 31]. However, the molecular mechanism of interaction between these proteins as well as functional significance of the signaling remain unclear. Thus, we also focused on the mechanism of interaction between N-terminal cytosolic tail of a2-subunit (a2N) of V-ATPase and cytohesin-2 (CTH2) [28]. The interaction sites between these two proteins were mapped using the combination of recombinant proteins/synthetic peptides pull-down and surface plasmon resonance (SPR) experiments. Two structural elements involved in specific and high affinity association of the a2 isoform with CTH2 were identified: (1) an N-terminal binding motif formed by the first 17 amino acids of the a2N (a2N1–17 peptide) and (2) the catalytic Sec7 domain of CTH2 [28, 31]. The SPR experiments further confirmed that these structural elements are major binding sites between a2N of V-ATPase and CTH2. Furthermore, this analysis revealed a strong binding affinity between this a2N1-17 peptide and the Sec7-domain of CTH2, with a dissociation constant of $K_D = 3.44 \times 10^{-7}$ M, similar to the binding affinity $K_D = 3.13 \times 10^{-7}$ M between full-length a2N and CTH2 proteins (Fig. 16.2, interfaces I and II). Analysis of enzyme activity confirmed that a2N1-17 peptide is crucial for V-ATPase/ CTH2 signaling and regulate the cytohesin-2 enzymatic Arf-GEF activity. Indeed, these studies revealed that a2N1-17 peptide is a potent inhibitor of the GDP/GTP-exchange activity of CTH2, that is acting through its direct interaction with the catalytic Sec7 domain. The α-helical structure of a2N1-17 and its residues F5, M10, Q14 binding with the Sec7 domain were also identified by NMR spectroscopy analysis (Fig. 16.4a, b). In silico docking studies have shown that a2N1-17 epitope of V-ATPase competes with the switch-2 region of Arf1,6 for binding to the Sec7 domain of CTH2 (Fig. 16.4c, d). Together, these experiments revealed the structural basis and molecular details of mechanism of signaling between V-ATPase and CTH2/Arf1,6 small GTPases (Fig. 16.4).

Although our previous work uncovered a functional cross talk between V-ATPase, cytohesin-2, and Arf1,6 small GTPases, other downstream effectors and related cell biological events have not been unraveled. However, since V-ATPase interacts with both cytohesin-2 and aldolase, we suggested that these proteins could in turn interact with each other, forming V-ATPase/cytohesin-2/Arf1,6/aldolase super-complex.
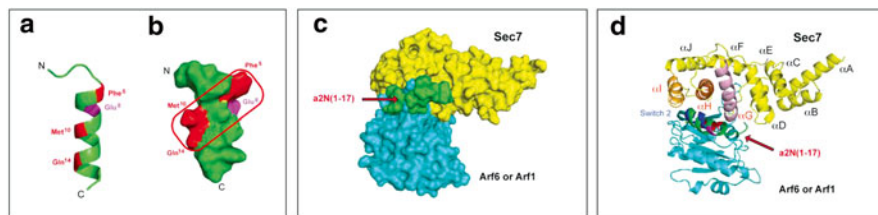
Fig. 16.4 The molecular features of the protein–protein interaction interface and signaling between V-ATPase and cytohesin-2/Arf1,6 small GTPases. (**a**) NMR structure $a2N(1-17)$ peptide derived from V-ATPase and mapping of its interaction protein–protein binding interface (formed by amino acids F5, M10, Q14, shown in *red square*) involved in binding with catalytic Sec7 domain of cytohesin-2. (**b**) In silico docking experiments of the a2N(1–17) peptide at the interface between catalytic site of the Sec7-domain of cytohesin-2 and Arf1,6 small GTPases. (**c**) The binding of the $a2N(1-17)$ peptide involves the αG, αH, and αI helices of Sec7 and switch 2 of the Arf1,6 small GTPases

The super-complex coordinates endocytic vesicle trafficking and downstream signaling of receptors (Fig. 16.3). The direct interaction of aldolase with cytohesin-2 through its PH-domain was shown by the pull-down and SPR experiments (Fig. 16.2). This approach revealed a two-step interaction between these two proteins with $K_{D1} = 1.1 \times 10^{-4}$ M and $K_{D2} = 2.7 \times 10^{-6}$ M, clearly indicating a potential regulatory mechanism of this interaction (Fig. 16.2, interfaces III and IV). Moreover, using a cell fractionation approach, we demonstrated the association of aldolase with early endosomes and formation of V-ATPase/cytohesin-2/Arf1,6/aldolase super-complex (Figs. 16.2 and 16.3). The aldolase knockdown experiments further uncovered the functional significance of interactions within V-ATPase/cytohesin-2/Arf1,6/aldolase super-complex. It was shown that the direct interaction between aldolase and cytohesin-2 are important in: (1) gelsolin gene expression, (2) actin cytoskeletal rearrangement, and (3) redistribution of endosomal vesicles within endocytic protein-degradation pathway [167].

In summary, a novel V-ATPase/cytohesin-2/Arf1,6/aldolase super-complex identified on early endosomes may be involved in regulation of the signaling receptors and function of the protein degradation pathway (Figs. 16.2 and 16.3). Moreover, these data indicate that pH-dependent binding and signaling between V-ATPase and cytohesin-2 may modulate the interaction of the a-subunit isoforms with aldolase and/or the GE-heterodimer forming peripheral stalks and consequently modulate the reversible association/dissociation of the V_1 and V_O of V-ATPase (Fig. 16.2) [1, 2]. Thus, evolutionarily acquired pH-sensing and cytohesin-2/Arf1,6-signaling function of V-ATPase, and its interaction with aldolase, may be an integral part of the self-regulatory mechanism of the enzyme as a proton-pumping rotary nano-motor.

On the other hand, downstream effectors of V-ATPase/cytohesin-2/Arf1,6/aldolase super-complex have also recently emerged. Cytohesin-1/2 family Arf-GEFs are shown to play role in regulation of signaling and trafficking of EGFR/ErbB and IR/IGF-1R receptors [128, 129, 154, 156, 157]. In particular, these studies demonstrated

the direct interaction of cytohesin-2 with insulin receptor and regulation of the PI3K signaling pathway. Thus, both in mice and fly cytohesin-2 is essential for signaling of IR/IGF-1R, cell growth, regulation of metabolism, and function of calorie restriction pathway (CRP) during aging process [154, 156, 157, 162, 164, 202]. In cancer cells, cytohesin-2 serves as a cytoplasmic activator of EGFR/ErbB, that modulates phosphorylation-dependent dimerization, oncogenic signaling of these receptors, and development of cancer (Fig. 16.3) [128, 129]. In conclusion, cell biological insights of V-ATPase/cytohesin-2/Arf1,6/aldolase super-complex (Figs. 16.2 and 16.3) shed light on the regulation of endocytic protein degradation pathway, trafficking and signaling EGFR/ErbB and IR/EGF-1R receptors under both physiological (longevity and aging process) and pathological (cancer and diabetes) conditions.

5 Conclusions

5.1 Drug Design and Targeting to Diverse V-ATPases and Their Super-complexes

As discussed above, extensive research in the last few years made an astounding breakthrough in understanding the structure and function of V-ATPase [1–5, 7, 9–12]. The advances include following discoveries: (1) low resolution structure of V-ATPase using cryo-EM; (2) the crystal-structure of individual subunits and their protein–protein interfaces; (3) endosomal V-ATPase/cytohesin-2/Arf1,6/aldolase super-complex; (4) lysosomal V-ATPase/Ragulator/Rag/mTORC1/Rheb super-complex, (5) novel roles of V-ATPase in trafficking and signaling of receptors, and (6) critical role of V-ATPases in the development and pathogenesis of human diseases. Thus, V-ATPase could be considered as a potential target providing powerful approaches for the development of therapeutic agents. The seven different approaches could be considered as discussed below.

First, targeting to V-ATPases in diverse compartments with a large spectrum of subunit isoforms could be productive for pharmaceutical research. The expressions of these isoforms are specific for tissues, cells, and compartments. Recent experiments demonstrated that V-ATPases with unique combinations of subunit isoforms are localized in specific cell membranes which could dictate their functions [1–5, 8]. Thus, isoform-specific subunits of V-ATPase have been suggested as attractive targets for the treatment of human diseases. Targeting a-subunit and other isoforms by small molecule inhibitors is proposed in treating lytic bone disorders [210–212]. V-ATPase a-subunit isoforms are also potential targets for the treatment of metastatic cancers. Thus, the small molecule V-ATPase inhibitors and siRNA have been studied extensively with their potential application in cancer treatment and prevention of metastasis [213–217].

Secondly, targeting the extracellular domains of a- and c-subunits with specific antibodies has been recently successfully applied for selective inhibition of plasma

membrane V-ATPase and reduction of metastatic phenotype of the cancer cells [53, 178]. In addition, the first V-ATPase inhibitory peptide was also identified and its selectivity was demonstrated [82]. Thus, this approach should be considered for developing of novel anticancer pharmaceuticals including antibodies and peptides which have high specificity and low toxicity.

Third, unique mechanism of regulating V-ATPase is reversible assembly/disassembly, which could be a promising target in drug design. This approach would be even more fruitful if combined with targeting cell or tissue specific V-ATPase isoforms [13–15, 19].

Forth, V-ATPases in regulation of signaling, trafficking, and degradation of cellular receptors could be a potential target. As discussed above, endocytosis, signaling, and degradation of Fz/LRP6, PRR, and Notch receptors are regulated by the direct interaction with V-ATPase [1, 122]. Therefore, targeting to the interactions between V-ATPase and these receptors could provide a promising approach in treatment of diseases related to cellular trafficking such as cancer, diabetes, and neurodegenerative diseases.

Fifth, regulatory proteins interacting with V-ATPase could be a potential target. The V-ATPase dependent super-complexes in early endosomes (V-ATPase/cytohesin-2/Arf1,6/aldolase) and late endosomes/lysosomes (V-ATPase/Ragulator/Rag/mTORC1/Rheb) pathway have been discovered recently in our [1, 2, 25, 26, 28, 30, 31, 109, 167] and Sabatini [32–34, 208] laboratories, respectively. The specific cascade of different protein–protein interactions within these complexes could modulate pH and amino-acids sensing, targeting, assembly, and activity of V-ATPase, linked to the regulation of intravesicular acidification and trafficking. For example, mTOR-signaling pathway is critical for the pathogenesis of the cancer and age-related diseases [34, 35, 163, 200–204]. It is noteworthy that extensive studies in animal models and clinical trials have uncovered the beneficial action of *rapamycin*, a potent mTOR inhibitor approved by FDA for treatment of variety age-associated diseases including cancers, neurodegenerative disorders, aging, and longevity. However, due to its side effects caused by the action of *rapamycin* on both mTORC1 and mTORC2, there is growing necessity for pharmacological research producing more specific and efficient drugs targeting of these pathways. Therefore, small molecules specifically targeting of protein–protein interactions in V-ATPase/Ragulator/Rag/mTORC1/Rheb super-complex will provide an attractive therapeutic agents to control aberrant signaling of mTORC1 and mTORC2 complexes in cancer, diabetes, age-related diseases, aging, and longevity [34, 35, 163, 200–204].

Sixth, novel endosomal V-ATPase/cytohesin-2/Arf1,6/aldolase super-complex and especially its protein–protein interaction interfaces could be a potential therapeutic targets (Figs. 16.2, 16.3, and 16.4) [1, 2, 25, 26, 28, 31, 167]. We propose that this approach will develop novel protein–protein interaction (PPI) inhibitors including therapeutic peptides or small molecule drugs [218–220]. These compounds may be also used to regulate assembly/disassembly of V-ATPase and signaling of cytohesin-2/Arf1,6 small GTPases. Modulation of the function of EGFR/ErbB or IR/IGF-1R receptors by the similar approach may be useful for treating variety of cancers, age-related diseases, slowing aging process and extending human longevity [154–158, 162–164].

Seventh, in our recent study the V-ATPase interactome was mapped for the first time. This systematic approach, has revealed a novel interacting proteins involved in trafficking, folding, assembly, and phosphorylation of V-ATPase [221]. These cell biological processes regulate V-ATPase-dependent acidification, and thus, these pathways and proteins could serve as potential drug targets for the therapeutic regulation V-ATPase function in health and disease states.

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